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PULMONARY ARTERIAL HYPERTENSION: A CORRELATION BETWEEN SEVERITY OF DISEASE AND NEW BIOMARKERS OF ENDOTHELIAL DAMAGE AND REPAIR.

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1 Summary in italian

L'ipertensione arteriosa polmonare è una condizione clinica che può manifestarsi in forma idiopatica o associata ad una varietà di patologie[1]. Anche se queste malattie presentano una differente eziologia, l'ipertensione polmonare in esse riscontrata è caratterizzata da tre predominanti meccanismi patobiologici che ne determinano l'epifenomeno clinico-patologico: l'attivazione di uno stato flogistico sistemico, una precoce disfunzione endoteliale ed una aumentata angiogenesi[2,3].

Un numero considerevole di evidenze scientifiche suggerisce il possibile coinvolgimento della flogosi sistemica nella patogenesi dell'ipertensione arteriosa polmonare. Elevati livelli circolanti di citochine infiammatorie (IL-1 ed IL-6), l'aumentata espressione polmonare di alcune chemochine (MIP-1α e MCP-1) e l' infiltrazione perivascolare di cellule infiammatorie (cellule T e macrofagi) è stata evidenziata in pazienti con ipertensione arteriosa polmonare[4-7].

Recentemente elevati livelli circolanti del CD40 ligando, una proteina trans-membrana implicata nello sviluppo di molte malattie infiammatorie come i disturbi autoimmuni, le reazioni da rigetto di

trapianto ed i processi aterosclerotici, sono stati riscontrati nei pazienti con ipertensione arteriosa polmonare[8].

Tuttavia ulteriori indagini sono necessarie per stabilire il ruolo dei meccanismi infiammatori nello sviluppo di tale patologia[2,3]. Primo scopo del nostro studio è stato quello di *valutare lo stato infiammatorio di tali soggetti così come appare dai principali indici bioumorali di flogosi.*

Sino ad oggi gli esami ematochimici non sono stati utilizzati clinicamente nello screening o nel follow-up dei pazienti con ipertensione polmonare; tuttavia diverse molecole (BNP, endotelina-1, acido urico, serotonina, NO, D-dimero, troponina T) sono elevate nel plasma di soggetti con tale patologia[9].

Si è pensato quindi di valutare se i livelli di alcuni comuni indici aspecifici di infiammazione, quali la conta dei globuli bianchi, la formula leucocitaria, la VES, i livelli di PCR, fibrinogeno ed acido urico fossero aumentati nei pazienti con ipertensione arteriosa polmonare, rispetto a controlli sani; abbiamo inoltre voluto valutare se esiste una correlazione tra l'aumento di questi indici di flogosi e la severità della malattia ipertensiva polmonare, valutata sulla base della classe NYHA e sulla distanza percorsa durante il 6MWT.

L'altro aspetto che il nostro studio ha considerato è stata la presenza degli indici di danno e riparazione endoteliale nei soggetti affetti da ipertensione arteriosa polmonare.

È consolidato in letteratura che nei pazienti con ipertensione arteriosa polmonare il danno all'integrità e alla funzione endoteliale (diminuita produzione di NO, di prostacicline e di sostanze antitrombotiche; ed aumentata produzione di trobassano A_2, di endotelina-1 e di sostanze pro-trombotiche[10]) portano a molteplici alterazioni nei normali processi omeostatici del sistema vascolare polmonare e determinano un rimodellamento importante della parete vasale attraverso l'instaurazione di un complesso circolo vizioso mediato dall'ipossia che ne consegue[11].

Il rimodellamento della parete arteriosa si caratterizza per un ispessimento determinato dall'accumulo di proteine della matrice extracellulare e dalla proliferazione e migrazione delle cellule muscolari liscie, stimolata dallo stravaso nella matrice extracellulare di mitogeni[12]. Recenti lavori su animali attribuiscono un ruolo importante nella patogenesi e nel mantenimento dell'ispessimento parietale alla proliferazione dei vasa vasorum avventiziali[13]. Le lesioni plessiformi che sono caratteristiche ma non patognomoniche

di ipertensione arteriosa polmonare costituiscono la forma più complessa di rimodellamento parietale[12].

In tale contesto fisiopatologico è nato il nostro interesse nel valutare nei soggetti affetti da questa malattia le concentrazioni sieriche delle microparticelle endoteliali e dei progenitori delle cellule endoteliali.

Infatti recentemente un considerevole interesse scientifico è stato rivolto alle microparticelle prodotte dallo sfaldamento della membrana citoplasmatica cellulare, come nuovo marker di danno endoteliale[14]. Un elevato livello plasmatico di microparticelle identificate principalmente dai cluster $CD31^{+}/CD42^{-}$ sono state riscontrate in soggetti con patologia del sistema cardiovascolare quale sindrome coronarica acuta, arteriopatia periferica e diabete mellito[13]. In tutte queste patologie le microparticelle endoteliali vengono considerate un indice di danno vascolare[14,15]. Un recente lavoro eseguito in pazienti con insufficienza renale severa ha mostrato che le microparticelle sono in grado di inibire il rilascio di ossido nitrico dalla cellule endoteliali suggerendo che esse non costituiscono solamente il risultato di un danno ma hanno un possibile ruolo nella genesi della disfunzione[16].

La scoperta nel sangue circolante di progenitori di cellule endoteliali[17] capaci di differenziarsi in multipli fenotipi cellulari (cellule muscolari lisce e cellule endoteliali) fa supporre che essi siano coinvolti nei meccanismi di angiogenesi e vasculogenesi dell'adulto nonché costituiscano un pool cellulare in grado di rigenerare il tessuto endoteliale perso e sostituire le cellule disfunzionanti[18], creando quindi una differente prospettiva al tradizionale concetto per cui si riteneva che fossero le cellule adiacenti ad una lesione a proliferare e re-endotelizzare l'esistente vasculatura[18].

Tuttavia discordanti sono i risultati degli studi che investigano se i progenitori partecipino solamente al mantenimento dell'omeostasi vascolare o intervengono anche nella patogenesi delle differenti patologie[18]. Nagaya et al[19] hanno mostrato, in studi su animali, che i progenitori delle cellule endoteliali somministrate per via endovenosa sono incorporate nelle arteriole polmonari e che se trasdotte con plasmidi contenenti adrenomedullina migliorano in maniera significativa il grado di ipertensione polmonare.

Uno studio preliminare recentissimo su 33 pazienti ha mostrato che l'infusione intravenosa di progenitori delle cellule endoteliali in soggetti con ipertensione arteriosa polmonare è praticabile e sicura

e che può avere effetti benefici sulla capacità funzionale e sulla emodinamica polmonare[20].

In questo contesto scientifico il nostro studio ha l'intento di valutare il rapporto tra le concentrazioni delle micropaticelle $CD31^{+}/CD42^{-}$ ed i progenitori delle cellule endoteliali in una popolazione di soggetti con ipertensione arteriosa polmonare idiopatica o associata correlando i valori ottenuti con gli indici strumentali di malattia.

In questo studio abbiamo quindi *valutato se esista una correlazione tra severità della patologia e disfunzione endoteliale stimata tramite dosaggio delle microparticelle* $CD31^{+}/CD42^{-}$ *, ed abbiamo correlato la severità della patologia con il numero di progenitori delle cellule endoteliali circolanti*.

CREAZIONE DATA BASE IPERTENSIONE POLMONARE E CORRELAZIONE TRA GRADING DELLA MALATTIA E STATO FLOGISTICO

La nostra ricerca ha avuto come primo intento quello di identificare i pazienti affetti da ipertensione polmonare, ricoverati al Policlinico di Padova, e rivalutare la loro diagnosi clinica sulla base della nuova classificazione elaborata nel corso del Congresso Internazionale di Venezia 2003.

Ciò ha permesso di constatare la casistica relativa a tale patologia afferita presso questo centro di terzo livello, creare un data base di pazienti aggiornato secondo la nuova nomenclatura, ed effettuare delle analisi sulla base dei dati recuperati.

Con tale fine abbiamo richiesto presso gli Uffici amministrativi dell'Azienda Ospedaliera di Padova l'elenco dei pazienti ricoverati al Policlinico Universitario dal 2000 al 2006 che presentavano come diagnosi di dimissione primaria o secondaria il codice DRG 4160 (ipertensione polmonare primitiva) od il codice 4168 (altre forme di ipertensione polmonare) della classificazione delle malattie ICD-9-CM[79].

La ricerca tramite codice DRG ha selezionato 255 cartelle. 35 di queste cartelle, un numero relativamente esiguo, pari al 14 % sono state escluse per incongruenze dei valori di pressione polmonare riportati all'esame ecocardiografico e\o invasivo e della patologia riportata alla dimissione con la diagnosi di ipertensione polmonare. L'analisi delle restanti 255 cartelle ha individuato 36 pazienti affetti da ipertensione arteriosa polmonare: nove pazienti risultavano affetti da ipertensione polmonare idiopatica e 27 da forme associate, di cui 11 a cardiopatia congenita, 5 ad una malattia del collagene, 8 ad AIDS, 3 ad ipertensione portale (2

affetti da cirrosi epatica ed 1 da sindrome di Budd-Chiari). Nel nostro data base il numero totale dei pazienti affetti da forme secondarie di ipertensione polmonare risultava di 182 soggetti, e sono stati esclusi.

Nella nostra analisi abbiamo considerato i pazienti con ipertensione arteriosa polmonare idiopatica o associata, appartenenti al primo gruppo della Classificazione Internazionale di Venezia del 2003, poiché il nostro obiettivo è stato correlare l'esistenza e l'entità della malattia ipertensiva polmonare con una attivazione umorale infiammatoria, indipendentemente che sia o non sia presente una patologia sistemica sottostante.

Dalla analisi dei dati rilevati dalle cartelle cliniche dei 36 pazienti affetti da ipertensione arteriosa polmonare idiopatica o associata e da quelli ottenuti in 36 soggetti sani, bilanciati per età e sesso con i primi, si è potuto rilevare quanto segue:

1. I pazienti affetti da ipertensione arteriosa polmonare avevano una conta di globuli bianchi (6700±113/mm^3 vs 6360±156/mm^3) e livelli di VES (21±2,5 mm vs 16±2,7 mm) sovrapponibili ai soggetti di controllo sani, tuttavia è emersa una differenza significativa ($p<0.05$) nei livelli di PCR (4.7±0,09 mg/l vs 1,6±0,04 mg/l), fibrinogeno (465±53 mg/dl vs 319±66 mg/dl), acido urico (5,1±0,8

mg/dl vs 3,2±0,7 mg/dl) tra i due gruppi; in particolare questi ultimi indici sono risultati essere significativamente più alti nei pazienti con ipertensione arteriosa polmonare. Al contrario i livelli di albumina sono risultati essere inferiori nei pazienti con ipertensione polmonare rispetto ai controlli sani (3.2±0,8 g/dl vs 4.1±1,1 g/dl,p=0,026).

2. Nei soggetti con ipertensione arteriosa polmonare, dal confronto dei livelli dei principali indici di flogosi nel gruppo di pazienti in classe NYHA III e IV vs quelli in classe NYHA I e II, sono emersi valori più alti dei parametri infiammatori nei soggetti nel primo gruppo: PCR (6.9±0,08 mg/l vs 2.8±0,05 mg/l), fibrinogeno (513±69 mg/dl vs 408±88 mg/dl) ed acido urico (7.6±1,1 mg/dl vs 2,6±1,4 mg/dl). Non sono emerse differenze significative nei livelli degli altri indici di flogosi presi in considerazione nel nostro studio tra i pazienti raggruppati nelle diverse classi NYHA.

3. Nei pazienti con ipertensione arteriosa polmonare, dalle analisi di correlazione tra indici di flogosi ed esito del 6MWT è emerso che i soggetti con minore distanza percorsa al 6MWT avevano livelli più alti di PCR (rho= -0.47, p=0.002), di fibrinogeno (r=-0.33, p=0.009) e di acido urico (r=-0.36, p=0.008). La VES, la conta dei globuli bianchi ed albumina non sono risultate essere

significativamente associate all'entità della limitazione funzionale dei pazienti con ipertensione arteriosa polmonare.

In conclusione possiamo affermare che i pazienti con ipertensione arteriosa polmonare presentano una attivazione sub-clinica della cascata infiammatoria, la quale potrebbe essere in relazione con il grado di compromissione della funzione vascolare polmonare.

Anche se l'incremento di comuni indicatori aspecifici di flogosi non rappresenta uno strumento dirimente per la diagnosi di ipertensione arteriosa polmonare, tuttavia, l'impiego di alcuni tra questi indicatori (PCR, fibrinogeno ed acido urico) potrebbe essere di una certa utilità nel migliorare la stratificazione del danno vascolare polmonare del paziente con ipertensione arteriosa polmonare oltre che consentire un più preciso inquadramento dello stadio della malattia vascolare polmonare.

Inoltre una serie di rilevanti considerazioni emergono dalla analisi dalla caratteristiche cliniche dei pazienti affetti da ipertensione polmonare inclusi nel nostro data base.

La sintomatologia presentata pone molti problemi di diagnosi differenziale con numerose altre patologie che presentano analoga manifestazione, quali la cardiopatia ischemica, lo scompenso cardiaco congestizio, le pneumopatie, la tromboembolia polmonare

cronica. La distinzione risulta estremamente difficile sulla base del racconto anamnestico e soltanto la positività dell'anamnesi familiare o patologica remota per le forme associate potrebbe suggerire il sospetto di tale malattia. L'altro dato rilevante, é che tali soggetti giungono alla diagnosi clinica tardivamente ed in fase avanzata della malattia. Ciò potrebbe essere attribuito alla necessità di un iter diagnostico che prevede numerose indagini strumentali come approccio alla sintomatologia riferita. Inoltre nelle forme associate il clinico sovente, tende ad interessarsi prevalentemente della patologia principale trascurando l'evoluzione ed il trattamento della malattia polmonare. Emerge quindi chiaramente che il sospetto clinico e la successiva diagnosi di ipertensione polmonare è di difficile elaborazione e costituisce essenzialmente una diagnosi di esclusione.

CORRELAZIONE TRA IPERTENSIONE POLMONARE ED INDICI DI DANNO E RIPARAZIONE ENDOTELIALE.

Il nostro studio è stato eseguito su di un gruppo di 15 pazienti affetti da ipertensione arteriosa polmonare (4 con IAP idiopatica, 5 con forma associata a collagenopatia, 5 con forma associata a cardiopatia congenita ed 1 caso di ipertensione polmonare associata ad ipertensione portale) e seguiti presso l'ospedale

Martini di Torino, l'ospedale provinciale Sant'Andrea di Vercelli e l'ospedale San Luigi Gonzaga di Orbassano. Tutti i pazienti hanno firmato consenso informato per la partecipazione allo studio. I soggetti in esame erano tutti in terapia con antagonisti recettoriali dell'endotelina. Tutti i pazienti avevano un anamnesi negativa per eventi cardiovascolari, dislipidemia, diabete, fumo ed ipertensione arteriosa. In tutti i pazienti un ecocolorDoppler dei tronchi sopraaortici ha mostrato valori normali di spessore medio-intimale ed il test di vasoreattività dell'arteria brachiale è risultato nella norma.

La popolazione in esame è stata confrontata con 15 soggeti sani bilanciati per sesso ed età con la popolazione affetta da ipertensione arteriosa polmonare.

Tutte le determinazioni sono state effettuate a digiuno, tra le 8:00 e le 10:00 del mattino. La misurazione del numero di microparticelle endoteliali è stata effettuata come descritto da Jimenez e coll.[69]. In breve, ogni paziente è stato sottoposto a prelievo venoso ed il sangue ottenuto è stato sottoposto a centrifugazioni sequenziali volte ad ottenere plasma povero in piastrine (PPP). Ogni campione di PPP è stato quindi incubato per 30 minuti a 4°C con anticorpi fluorescenti: anti-CD31 coniugato con

il fluorocromo *ficoeritrina* (PE) e anti-CD42a coniugato con il fluorocromo *fluoresceina isotiocianato* (FITC). Trascorso tale tempo su ogni campione è stata eseguita un'analisi citofluorimetrica volta all'individuazione della sottopopolazione delle MPs caratterizzata da: presenza dell'antigene 31 sulla superficie della membrana, assenza dell'antigene 42a e dimensione inferiore a 1,5 μm. Tale operazione è stata condotta eseguendo un "gate" dimensionale mediante l'utilizzo di sfere di calibrazione per citofluorimetro a diametro noto.

Per la misurazione del numero di progenitori endoteliali, ad ogni partecipante allo studio è stato prelevato un campione di sangue in provette con EDTA come anticoagulante. Ciascun campione di sangue intero è stato sottoposto a centrifugazione in gradiente di densità allo scopo di separare le cellule mononucleate (linfociti e monociti). Dopo lavaggi sequenziali con PBS, volti ad eliminare l'eventuale contaminazione piastrinica, le cellule ottenute sono state incubate a 4°C per 30 minuti con anticorpi anti-CD34-FITC ed anti-KDR-PE. Dopo tale tempo su ogni campione è stata eseguita un'analisi citofluorimetrica volta all'individuazione dei progenitori endoteliali caratterizzati dalla co-espressione degli antigeni CD34 e KDR. Le analisi statistiche sono state effettuate con SPSS 15.0. La significatività statistica è considerata per valori di $p<0.05$.

Dal nostro studio emerge che i pazienti con ipertensione arteriosa polmonare hanno un maggior numero/µl di microparticelle endoteliali (835.8±15.7 *vs* 710.9±31.7; p<0.01) ed un minor numero/ml di progenitori endoteliali (423.9±7.4 *vs* 498.2±26.2; p<0.01), rispetto ai controlli sani. L'analisi di correlazione tra il numero di microparticelle endoteliali ed i livelli di PAPs determinata con esame ecocardiografico ha mostrato che esiste una relazione significativamente positiva tra queste due variabili (r=0.609; p=0.004). Inoltre la correlazione tra numero di progenitori circolanti delle cellule endoteliali e valori di PAPs hanno mostrato una associazione inversa (r=-0.463; p=0.05).

Dall'analisi di correla ne tra numero di microparticelle e valori del 6 MWtest è emerso che esiste una associazione tra le suddette variabili in quanto al diminuire della distanza percorsa si verifica un incremento del numero di microparticelle (r=-0.573, p=0.001). Al contrario il numero dei progenitori delle cellule endoteliali diminuisce con il ridursi dei risultati del 6MW test (r=0.444; p=0.014).

Conclusioni

L' equilibrio tra danno endoteliale e riparazione dell'endotelio danneggiato è essenziale per il mantenimento dell'omeostasi

vascolare. Dai risultati del nostro studio riteniamo che le microparticelle endoteliali $CD31^{+}/CD42^{-}$ possano rappresentare un nuovo valido indicatore del danno prodotto a livello dell'albero vascolare polmonare. D'altra parte, sembra emergere sempre con maggiore evidenza che il danno endoteliale rappresenti una condizione essenziale ma non sufficiente per deteriorare in modo significativo la funzione vascolare. Affinché ciò possa realizzarsi, si ritiene indispensabile che al danno vascolare si associ un'alterazione dei meccanismi di riparazione del danno stesso.

Oggi, si pensa che i progenitori endoteliali possano assolvere alla funzione di riparazione del danno endoteliale; pertanto, tutte le condizioni capaci di influenzare il numero o la funzione dei progenitori endoteliali possono contribuire in modo significativo alla riparazione del danno endoteliale o alla disfunzione vascolare.

I risultati del nostro studio hanno dimostrato che l'ipertensione arteriosa polmonare può essere associata in modo rilevante a questi due importanti fenomeni, il cui corretto equilibrio garantisce la normale funzione vascolare; in altre parole, l'ipertensione arteriosa polmonare, associandosi ad una frammentazione delle cellule endoteliali mature, incrementando la formazione di microparticelle endoteliali e riducendo il numero e la vitalità dei

progenitori endoteliali circolanti, costituisce l'espressione funzionale di una disfunzione vascolare in grado di determinare un rimodellamento strutturale e biologico dell'albero vascolare polmonare e di interressare complessi meccanismi omeostatici sistemici.

2 Summary in English

Pulmonary arterial hypertension (PAH) is a clinical condition that can present in either an idiopathic form or associated with a variety of other conditions[1]. Pulmonary arterial hypertension is characterized by three predominant pathobiological mechanisms that influence the clinical-pathologic phenomenon: the activation of a systemic inflammatory reaction, a premature endothelial dysfunction and an increased angiogenesis[2,3]. However, further investigations are needed, to establish the role of inflammatory mechanisms in the development of PAH. *The principal goal of this study, was to evaluate the inflammatory state of such pathologies specifically by examining the inflammatory profile in these subjects.* To date, blood test indexes have not been used clinically in either screening or follow-up of PAH patients; stating from the fact that several molecules (BNP, endotelin-1, uric acid, serotonin, NO, D-dimer, troponin T) are elevated in these patients[9]. We proposed an investigation looking at plasma levels of several inflammatory indexes in PAH patients. We studied white blood cell count and formula, ESR, CRP levels, fibrinogen and uric acid levels. Furthermore we wanted to find out if there was a correlation

between an increase in these phlogistic indexes and the severity of PAH based upon the NYHA classification and the 6MWT.

Another aim of this study was to establish the presence of endothelial damage and repair indexes in PAH subjects. CD31+/CD42- micro-particles and endothelial cell progenitors are now considered new markers for endothelial dysfunction. The second part of our study was to determe a possible relationship between CD31+/CD42- micro-particles and endothelial progenitor cells in a population of subjects with idiopathic or associated PAH. To do this, we compared the plasma levels of CD31+/CD42- micro-particles and endothelial progenitor cells with diagnostic indexes of disease (echocardiographic PAPs, 6MWT). *In conclusion, this study investigated a possible correlation exist between the severity of pathology and endothelial dysfunction estimated by CD31+/CD42—microparticle and endothelial progenitor cells count.*

THE CREATION OF A PULMONARY HYPERTENSION DATABASE AND CORRELATION BETWEEN DISEASE GRADING AND INFLAMMATORY BIOMARKERS

Aim of our research was to identify patients affected by pulmonary hypertension (PH), admitted to the University Hospital of Padua and re-evaluate their clinical diagnoses on the basis of the

new classifications adopted at The 2003 World Symposium on Pulmonary Arterial Hypertension held in Venice, Italy.

According to this new classification we have created a new database of patients and we have analyzed the collected data accordingly.

We have obtained from University of Padua, the lists of patients admitted between 2000 and 2006 and discharged with the code of the disease classification ICD-9-CM[79] 4160 (primary pulmonary hypertension) or 4168 (other forms of pulmonary hypertension).

We have selected 255 cases. Thirty-five (14%) were excluded for incongruence between the values of pulmonary pressure that were obtained from echocardiographic and/or invasive examination and diagnosis reported at discharge. The analysis of the cases identified 36 patients affected by PAH: 9 with idiopathic PAH and 27 with associated form of PAH (which 11 presented congenital cardiopathy, 5 collagen disease, 8 AIDS, 3 portal hypertension). In our database the total number of patients affected by other forms of PH was 184 subjects.

Since we limited our research to forms of PAH, patients with other forms were excluded.

In our analyses we considered patients with all the forms of pulmonary hypertension, which were included in the first group of the International classification held in Venice 2003. The aim of this was to correlate the existence and the severity of the pulmonary damage to inflammatory indexes, either in the absence or presence of any underliyng systemic disease.

The results relative to the medical charts of the 36 patients with idiopathic or associated PAH and the data obtained from the control group of 36 healthy subjects, the following was revealed:

1) the patients affected by PAH compared to healthy control subjects had white blood cell counts of 6700±113/mm^3 vs 6360±156/mm^3 and ESR levels of 21±2.5 mm vs 16±2.7 mm respectively. However, a significant difference emerged ($p<0.05$) in the CRP (4.7±0.09 mg/l vs 1,6±0.04 mg/l), fibrinogen (465±53 mg/dl vs 319±66 mg/dl), uric acid (5,1±0.8 mg/dl vs 3,2±0.7 mg/dl) levels between the two groups; all these indexes were significantly higher in patients with pulmonary arterial hypertension. On the other hand, the levels of albumin were inferior in patients with pulmonary arterial hypertension compared to healthy controls (3.1±0.8 g/dl vs 4.1±1.1 g/dl, $p=0.026$).

2) In subjects with pulmonary arterial hypertension comparing the principal inflammatory indices levels between the combined group of patients in the NYHA III and IV classes vs those subjects in the NYHA classes I and II, emerged higher levels for all inflammatory parameters in the first group: CRP (6.9±0.08 mg/l vs 2.8±0.05 mg/l), fibrinogen (513±69 mg/dl vs 408±88 mg/dl) and uric acid (7.6±1.1 mg/dl vs 2.6±1.4 mg/dl). Non significant differences were observed in the other inflammatory indexes examined in our study according to NYHA classes.

3) In subjects with pulmonary arterial hypertension the correlation analysis between the inflammatory indexes and the results at 6MWT showed that the subjects with decreased exercise capacity had higher levels: CRP (r= -0.47, p=0.002), fibrinogen (r=-0.33, p=0.009) and uric acid (r=-0.36, p=0.008).

In conclusion, we can affirm that patients with PAH present a sub-clinical activation of the inflammatory cascade; this could be related to the severity of pulmonary vascular damage. Even if the increase in the common non-specific inflammatory indicators does not represent a diriment tool for the diagnosis of PAH, the use of several of these markers (CRP, fibrinogen and uric acid) could be useful in improving the stratification of pulmonary vascular damage

in patients with PAH. Moreover, these indexes could provide a more precise assignement for pulmonary vascular disease staging. Furthermore, a series of relevant observations has emerged from the analysis of clinical characteristics in patients with PH included in the database: 1)the clinical presentation poses many problems regarding differential diagnoses and 2) these subjects had clinical diagnosis in the advanced phase of disease.

THE CORRELATION BETWEEN PULMONARY ARTERIAL HYPERTENSION AND NEW INDEXES OF ENDOTHELIUM DAMAGE AND REPAIR.

Our study, was carried out in Italy on a group of 15 patients affected by PAH from the Martini Hospital in Turin, the Sant'Andrea Hospital in Vercelli and the San Luigi Gonzaga Hospital in Orbassano. All patients had a negative history for cardiovascular disease, dyslipidemia, diabetes, smoke and systemic hypertension. A carotid echo-Doppler scan was performed on all patients and showed normal intimal-medial thickness values. A brachial arterial flow-mediated vasodilation after reactive hyperemia was assessed by ultrasonography and was normal in all patients. Fifteen PAH affected patients were compared with 15 healthy subjects matched

for sex and age. The measurements of the endothelial microparticles were performed as described by Jimenez e coll.[39] To determine the number of endothelial progenitor cells, a blood sample was taken (using the anticoagulant EDTA) in all participants in the study. Each sample of whole blood underwent a centrifugation on density gradient to separate the mononuclear cells (lymphocytes e monocytes). After sequential washing with PBS, to eliminate the eventual platelet contamination, the obtained cells were obtained were incubated at 4°C for 30 minutes with antibodies anti-CD34-FITC and anti-KDR-PE. After that, each sample underwent a cytofluormetric analysis to count endothelial progenitor cells expressing CD34 and KDR.

This study revealed that patients with PAH have a higher number/µl of endothelial microparticles (835.8±15.7 *vs* 710.9±31.7; $p<0.01$) and a lower number/ml of endothelial progenitors (423.9±7.4 *vs* 498.2±26.2; $p<0.01$), compared with the healthy control group.

The correlation analysis between the number of endothelial microparticles and the PAP levels measured with echocardiography demonstrated a significantly positive relationship between these two variables ($r=0.609$; $p=0.004$).Furthermore, the was a negative correlation between the number of circulating progenitors of the

endothelial cells and the PAP values (r=-0.463; p=0.05). The correlation between the number of microparticles and the 6MW test showed, an association between these variables. In fact, when exercise capacity was decreased, the number of microparticles was increased (r=-0.573, p=0.001). On the other hand, when the number of endothelial progenitor cells decreased, there was also a reduction in the 6MW test (r=0.444; p=0.014).

In conclusion the balance between endothelial damage and repair is essential for the maintenance of vascular homeostasis. We think that the endothelial microparticles $CD31^{+}/CD42^{-}$ represent a new biomarker of damage pulmonary vasculature damage. On the other hand the endothelial damage is a condition essential, but not sufficient for the deterioration of vascular function, which recognizes an alteration in the repair mechanisms. The results of this study demonstrate that PAH is associated with these two important conditions, which together guarantee normal vascular function. In other words, PAH is associated with a fragmentation of mature endothelial cells, increased formation of endothelial microparticles which in turn reduces both the number of circulating endothelial progenitor cells. This constitutes the functional expression of a vascular dysfunction that determines a structural

and biological reshaping of the pulmonary vascular tree that also involves complex systemic homeostatic mechanisms.

PULMONARY ARTERIAL HYPERTENSION: A CORRELATION BETWEEN SEVERITY OF DISEASE AND NEW BIOMARKERS OF ENDOTHELIAL DAMAGE AND REPAIR

3. INTRODUCTION

Pulmonary arterial hypertension (PAH) is a clinical condition that can manifest itself in either an idiopathic form or can be associated with a variety of other pathologies[1].

Even if these diseases present different aetiologies, the underlying underlying structural features are characterised by three predominant pathobiological mechanisms: the activation of one inflammatory systemic reaction, a premature endothelial dysfunction and an increased angiogenesis[2,3].

A considerable number of scientific evidence presently suggest the possible involvement of the systemic inflammation in the pathogenesis of PAH. Elevated levels of circulating inflammatory cytokines (IL-1 ed IL-6), increased pulmonary expression of several chemokines (MIP-1α e MCP-1) and perivascular inflammatory cells infiltration (T-cell and macrophages) have been observed in patients with PAH[4-7].

Recently, elevated levels of circulating ligand CD40, a trans-membrane protein involved in the development of many auto-immune diseases disturbances, transplant rejection as well as atherosclerosis, have been observed in patients with PAH[8].

Further investigations are needed to establish the role of inflammatory mechanisms in the development of these diseases. *Aim of this study, was to evaluate the inflammatory state of PAH looking at the bioumoral indexes of inflammation.*

To date, biochemical markers have not been utilised clinically in either the screening or the follow-up of PAH patients; despite the fact that diverse molecules (BNP, endotelin-1, uric acid, serotonin, NO, D-dimer, troponin T) are elevated in patients with these pathologies[9].

For this reason, further investigation was proposed to establish if the levels of several common indexes aspecific for inflammation were increased in PAH patients compared to healthy subjects. We considered white blood cell count and formula, ESR, CRP levels, fibrinogen and uric acid levels. Furthermore, this study was undertaken to determine if there was a correlation between an increase in these inflammatory indexes and the severity of PAH based upon the New York Heart Association (NYHA) classification and the distance travelled during the 6MWT.

Another aim of this study was to establish the presence of endothelial damage and repair processes in PAH subjects.

Patients with PAH have endothelial dysfunction (diminished production of NO, prostacycline and antithrombotic substances and an increased production of trobossan A_2, endotelin-1 and pro-thrombotic substances[10]). This causes alterations of the normal haemostatic processes of the vascular system and important remodelling of the vessel walls through the establishment of a complex vicious circle mediated by subsequent hypoxia [11].

The remodelling of the arterial walls is characterised by a thickening caused by accumulation of extra-cellular proteins and proliferation and migration of smooth muscle cells which are stimulated by the spillage of mitogens in the extra-cellular matrix[12].

Recent work carried out on animals have attributed an important role to the adventitial vasa vasorum in the pathogenesis and the maintenance of the wall thickening[13].

The plexiform lesions that are characteristic but not pathognomonic of PAH constitute the most complex form of wall remodelling[12].

This led us to investigate the serum concentrations of endothelial microparticles and endothelial cell progenitors in subjects with PAH.

We in fact think that endothelial microparticles, which are produced by the shedding of cytoplasmatic cellular membranes, may be a new markers for endothelial damage[14].

Patients with acute coronary syndrome, peripheral arteriopathy and diabetes mellitus[13] have an elevated levels of $CD31^{+}/CD42^{-}$ micro-particle.

In these pathologies the endothelial micro-particles are considered an index for vascular damage[14, 15].

A study carried out in patients with severe renal insufficiency has demonstrated that the micro-particles are capable to inhibit the release of NO from endothelial cells, suggesting that the micro-particles may also have a role in the genesis of endothelial dysfunction[16].

The discovery in the circulating blood of endothelial progenitors cells[17] which are capable of differentiate into multiple cell phenotypes (smooth muscle cells and endothelial cells) has suggested that they may be involved in the mechanisms of angiogenesis and vasculogenesis. They can also constitute a possible cell pool capable of regenerating endothelial tissue and substituting dysfunctional cells[18]. In this way, a new prospective as been created contrary to the previous theory holding that the cells adjacent to a lesion are responsible for the proliferation and regeneration of the endothelial layer of the existing vasculature[18].

Even so, there is little agreement whether progenitors only participate in the maintenance of vascular homeostasis or also intervene in the pathogenesis of different disease[18]. Nagaya et. al have demonstrated in animal studies that endothelial progenitor cells administered intravenously are incorporated in the pulmonary arterioles and if transduced with plasmids containing adrenomedullin, the grade of pulmonary hypertension improves significantly[19].

A very recent preliminary study on 33 patients has shown that the intravenous infusion of endothelial progenitor cells in subjects with PAH is feaseble and safe and may have beneficial effects on pulmonary haemodynamics[20].

In this scientific context this study intented to establish a possible relationship between $CD31^{+}/CD42^{-}$ micro-particles and endothelial cell progenitors in a population of subjects with idiopathic or associated PAH. To do this, we compared the obtained plasmatic levels of $CD31^{+}/CD42^{-}$ micro-particles and endothelial cell progenitors with diagnostic indexes of disease (echocardiographic PAPs, 6MWT).

We specifically looked to a possible correlation between the severity of the disaese and endothelial dysfunction estimated by circulating

levels of $CD31^{+}/CD42^{-}$ microparticles and endothelial progenitor cells.

4.1 THE DEFINITION AND CLASSIFICATION OF PULMONARY HYPERTENSION ACCORDING TO THE 2003 WORLD SYMPOSIUM IN VENICE, ITALY.

Pulmonary hypertension (PH) is a clinical condition that includes different pathologies characterized by increased systolic pressure in the pulmonary artery greater than 35 mmHg or mean pressure greater than 25 mmHg at rest or 30 mmHg after exercise[21].

During the course of the 2003 World Symposium on PAH the decision was made to update the existing classification, which had been formulated at the 1998 Symposium in Evian.

The main purpose of the new classification was to identify clinical conditions, which in the development of an increased pressure in the pulmonary circulation, would present common elements regarding physiopathologic mechanisms, histological lesions, clinical presentation and response to therapy[22].

The revised classification (Table 1) maintains the framework of the previous classification, distinguishing the diverse forms of PH into five classes: the first includes PAH (pulmonary artery hypertension), the second includes the form secondary to valvular cardiopathy or PH with left heart disease , the third includes the

form secondary to lung disease and/or chronic hypoxemia, the fourth includes PH due to thrombotic and/or embolic disease while the fifth includes rare diseases of pulmonary vessels[22].

In the new classification the term "Primary PAH" replaced the term "Idiopathic PAH". Previously, the Evian classification had changed the term "Secondary PAH" into "Associated PAH" , in consideration of the heterogeneous nature of the included diseases.

Therefore, the revised classification distinguishes PAH as an idiopathic form, a familial form and forms associated to various pathologies: collagen disease, congenital cardiopathy with systemic-pulmonary shunts, portal hypertension, HIV infection and other rare disorders.

Due to the analogy of the histological reports and similar clinical presentations, two pathologies associated with an involvement of the venular and capillary circulation(pulmonary veno-occlusive disease and pulmonary capillary hemangiomatosis) were included among the forms of PAH. In the fifth class the new classification considered: sarcoidosis, hystiocytosis X, lymphangiomatosis and compression of pulmonary vessels (adenopathy, tumour and fibrosing mediastinitis).

The Venice classification clarify that the term PAH refers to the clinical situations determined by a structural alteration of the parietal walls of pre-capillary arteries by means of pathological remodelling. Thus, it exclude form of dynamic hypertension or secondary to pulmonary venous osbtraction[22].

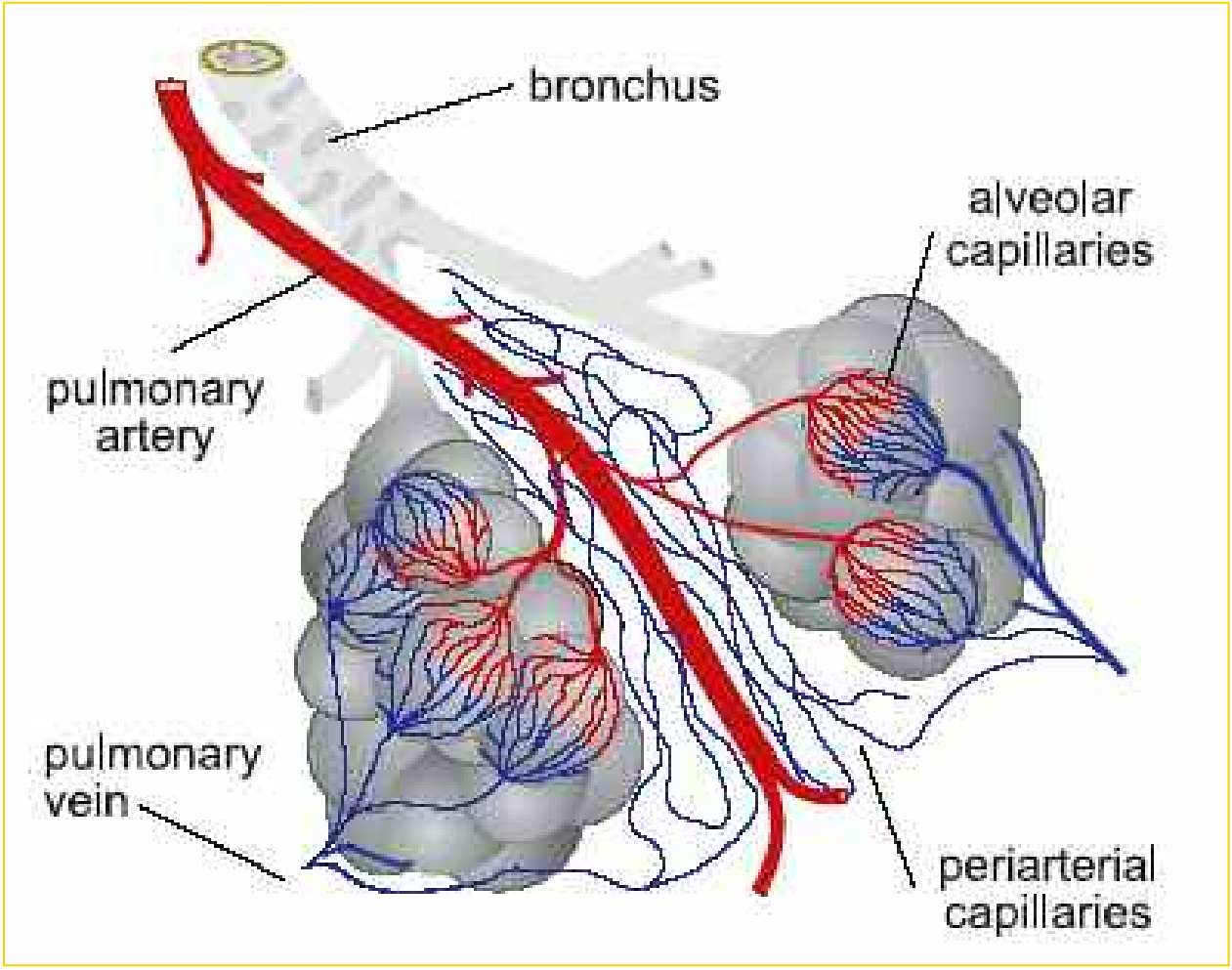
Table 1 Classification of pulmonary arterial hypertension according the 2003 world symposium in Venice, Italy

1 Pulmonary Arterial Hypertension
1.1 Idiopathic
1.2 Familial
1.3 Related to:
1.3.1 Collagen vascular diseases
1.3.2 Congenital heart diseases with systemic to pulmonary shunts
1.3.3 Portal hypertension
1.3.4 HIV infection
1.3.5 Pharmacological products and toxins
1.3.6 Other conditions (thyroid gland dysfunction, glycogen deposit disease , Gaucher's Disease, hereditary haemorrhages telangectasis, haemoglobinopathy, mieloproliferative disorders, splenectomy)
1.4 Associated with significant venal and capillary involvement:
1.4. Pulmonary veno-occlusive disease
1.4.2 Pulmonary capillary hemangiomatosis
1.5 Persistent PH of the newborn

2 ***Pulmonary Hypertension with Left Heart Disease***
2.1 Left ventricular dysfunction
2.2 Vavular dysfunction
3 PH with Lung Diseases/Hypoxemia
3.1 COPD
3.2 Interstitial lung diseases
3.3 Sleep disordered breathing
3.4 Alveolar hypoventilation
3.5 Chronic exposure to elevated altitudes
3.6 Developmental abnormalities of the respiratory system
4 PH due to Chronic Thrombotic and/or Embolic Disease
4.1 TE obstruction of proximal PA
4.2 TE obstruction of distal PA
4.3 Non thrombotic pulmonary embolism (tumours, parasites, foreign bodies)
5 Miscellaneous**.** Sarcoidosis, Hystiocytosis X, lymphangiomatosis, compression of pulmonary vessels of the pulmonary vessels of adenopathy, tumours, fibrosing mediastinitis.

4.2 PATHOLOGICAL CLASSIFICATION OF VASCULOPATHIES OF PULMONARY HYPERTENSION

The pathological classification of vasculopathies of pulmonary hypertension is reported here from the guidelines of the European Society of Cardiology and is based on the topographic distribution involvement of lesions according to the pulmonary tree segmentation.



Pathological Classification

(1) **Pulmonary arteriopathy** (pre-and intra-acinar arteries)

Subsets

-Pulmonary arteriopathy with isolated medial hypertrophy

- Pulmonary arteriopathy with medial hypertrophy and intimal thickening (cellular, fibrotic)

– Concentric laminar

– Eccentric, concentric non-laminar

-Pulmonary arteriopathy with plexiform and/or dilatation lesions or arteritis

- Pulmonary arteriopathy with isolated arteritis

(1a) As above but with coexisting venous-venular changesa (cellular and/or fibrotic intimal thickening, muscularisation)

(2) **Pulmonary occlusive venopathy** (veins of various size and venules) with or without coexisting arteriopathy

(3) **Pulmonary microvasculopathyc with or without coexisting arteriopathy and/or venopathy**

(4) **Unclassifiable**

Since we concentrated our attention on pulmonary arteriopathy we reported here a detail description of the pathological substrates, taken from the guidelines of European Society of Cardiology.

The main histopathological features of pulmonary arteriopathy include medial hypertrophy, intimal thickening, adventitial thickening and complex lesions.

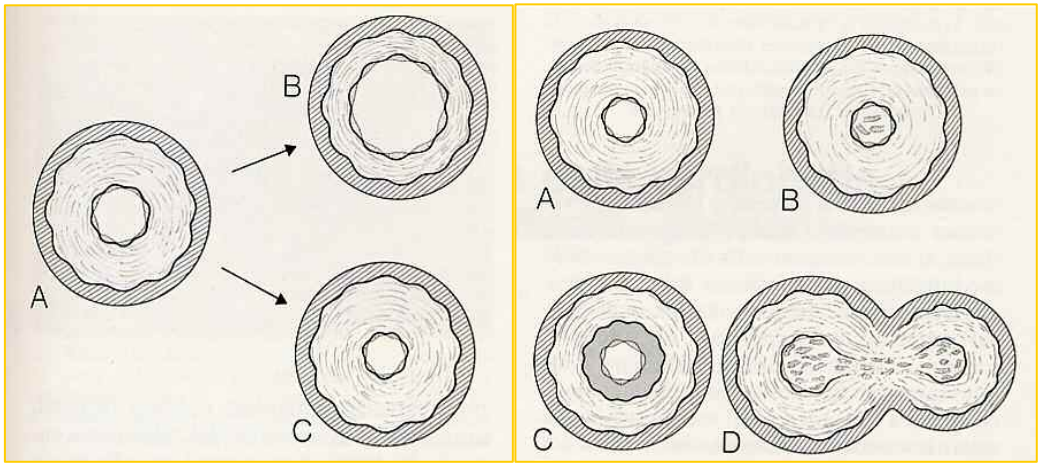
Medial hypertrophy is an increase in the cross sectional area of the media of pre and intra-acinar pulmonary arteries. It is due to both hypertrophy and hyperplasia of smooth muscle fibers as well as increase in connective tissue matrix and elastic fibers in the media of muscular arteries.

Intimal thickening may be concentric laminar, eccentric or concentric non-laminar. Ultrastructurally and immuno-histochemically the intimal cells show features of fibroblasts, myofibroblasts and smooth muscle cells.

Adventitial thickening occurs in most cases of PAH but it is more difficult to evaluate.

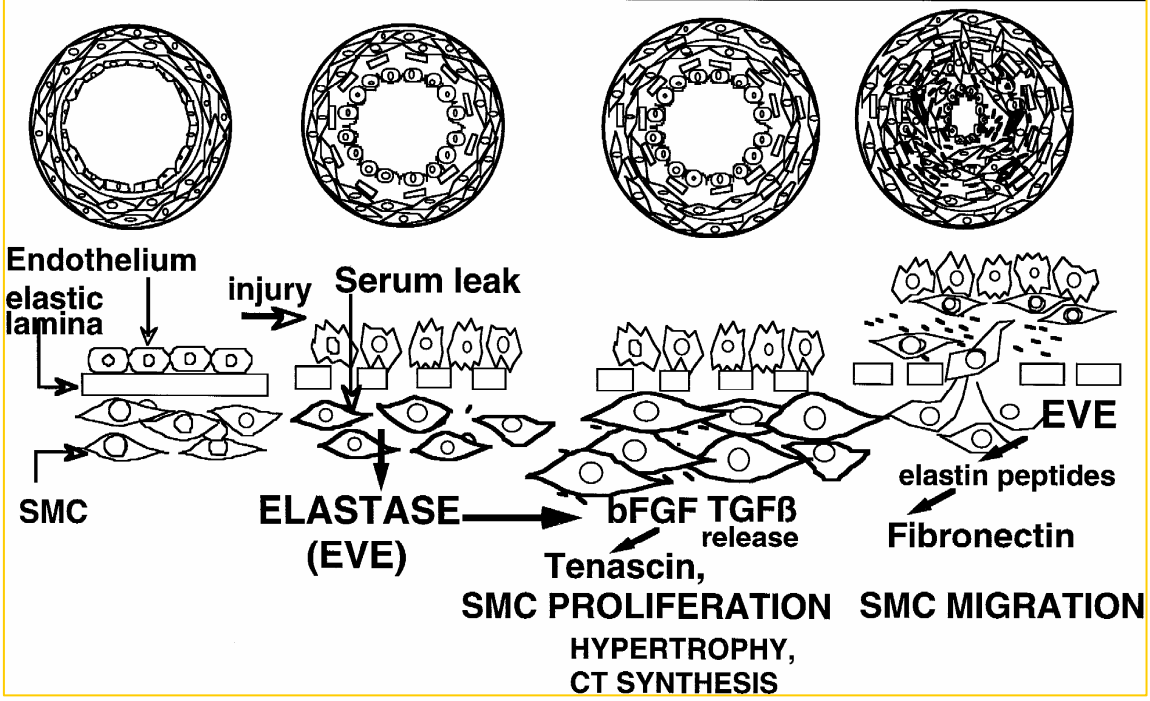
Complex lesions. The plexiform lesion is a focal proliferation of endothelial channels lined by myofibroblasts, smooth muscle cells and connective tissue matrix. These lesions are at an arterial branching point or at the origin of a supernumerary artery, distally

to marked obliterative intimal thickening of the parent artery. The frequency of the plexiform lesions in PAH remains undetermined. Arteritis may be associated with plexiform lesions and it is characterised by a necrosis of the arterial wall with fibrinoid insudation and infiltration with inflammatory cells.



Scheme of the type of lesions in pulmonary arteriopathy

Pulmonary Vascular Disease and Elastase Activity



Rabinovitch M, Cardiovasc Res 1997:34; 268-272

5. PATHOBIOLOGICAL MECHANISMS OF PULMONARY ARTERIAL HYPERTENSION

From a pathological point of view, vascular PAH occurs prevalently in vessels of a small diameter (<500μm) and in all of the arterial wall layers characterized by hypertrophy and hyperplasia of the endothelial cells, fibroblasts and smooth muscle cells and deposits of collagen, elastin and fibronectine (fig 1)[23].

The disorganised proliferation of the endothelial cells in a stroma formed by proteins of an extracellular matrix and miofibroblasts produce plexiform lesions (fig 1), which constitute the histological lesions characteristic of PAH, even if they are not pathognomic in any form [23]. It has been demonstrated in several studies that the plexiform lesions are formed by a population of monoclonal endothelial cells suggesting the existence of a primary defect in the regulation of such cells[23].

While the hyperplasia of the smooth muscle cells, on the other hand, results in the extension of the muscle cells in small vessels normally non-muscularised, which are the pre and intra-acinar arterioles [23].

Fibroblasts of the adventitial layer in severe PAH forms proliferate and migrate and contribute, through the deposit of the extracellular matrix, to the formation of a layer called neointima, between the endothelium and the internal elastic lamina[23].

Many factors are involved in the remodeling of the arterial wall. Currently, the objects of intense research activity include the vessel-active mediators: prostacycline, VIP, nitric oxide, endothelin, ion channels , endothelial growth factors (VEGFR-A and VEGFR-B) , and the cytokines (activine and TGF-βs) [23]. All of these appear to act through: the proliferation and the dysfunction of the endothelial cell, the thrombotic obliteration of the vascular lumen, the alteration of the complex control mechanisms of the vascular tone, the deposit of collagen and extracellular matrix, the inhibition of apoptosis, and the modulation of the inflammatory response [23]. Recently, the mutation of the gene regulating the transcription of the bone morphogenetic protein receptor type II" (BMPR2) has been documented in around 50% of familial PAH cases and about 26% in the sporadic forms[24]. The progress made in understanding the molecular mechanisms of this pathology, favoured the introduction of new pharmacological drugs that improved the negative outcome of these patients[25].

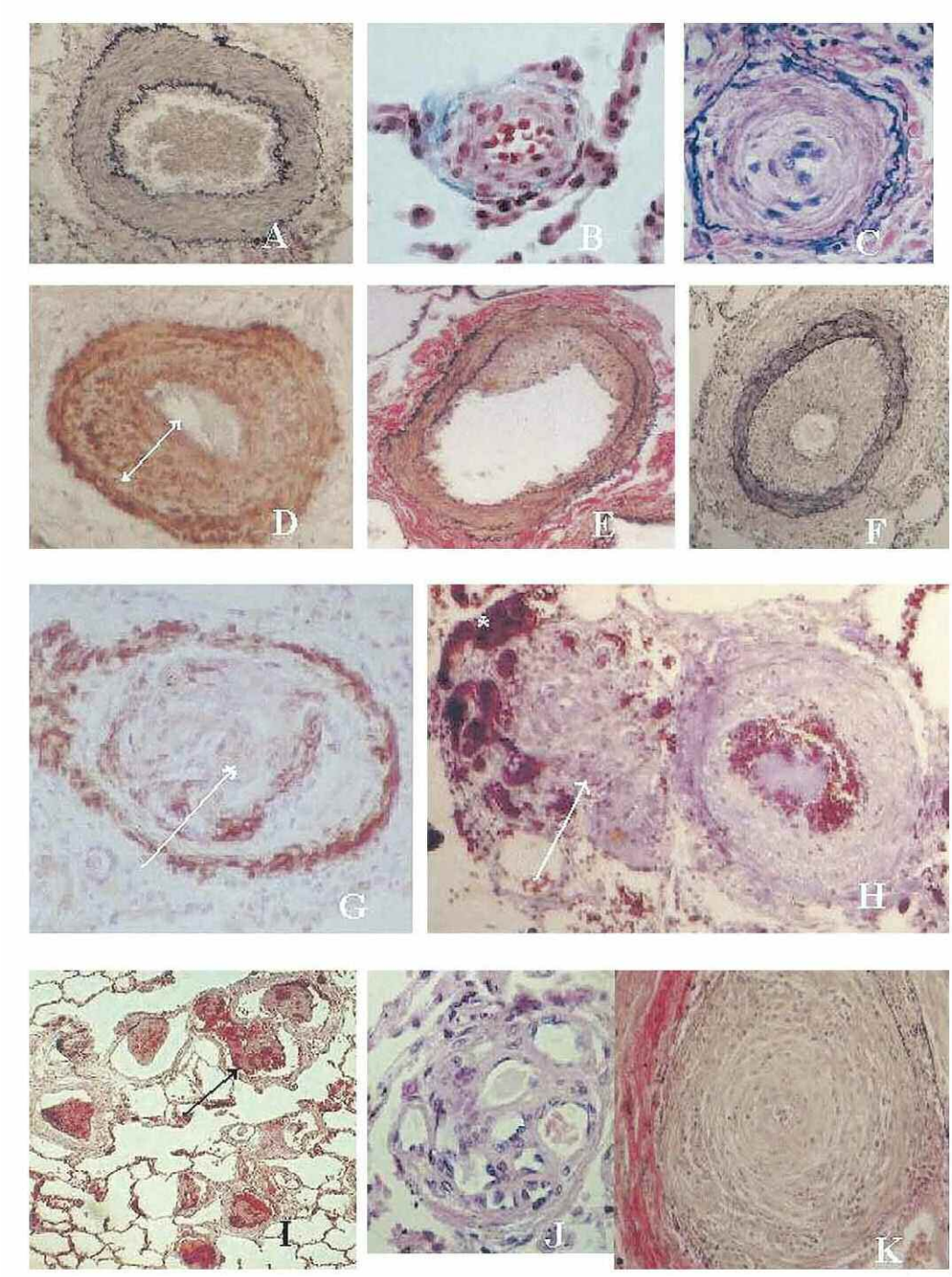


Fig.1. Principal histopathologic stages of pulmonary arteriopathy. Hypertrophy of the medial tunic: A)a. pulmonary preacinar, B)a. intracinar. Concentric intimal thickening of the internal lamina:C)a. intracinar, D)a. preacinar. Intimal thickening of the preacinar artery of an eccentric type (E) and concentric non laminar(F). Plexiform lesions in a. preacinar (G).A. preacinar adjacent to a plexiform lesion(H). Dilated lesions (I e J). Lymphomonocytic arteritis (K). From JACC 2004;43:12-23.

6. ENDOTHELIAL PROGENITOR CELLS

Stem cells can be defined as non-specialised cells, with a clonogenic or replicating potential, capable of auto-renovation and able to differentiate into diverse cellular lines. The division produces two cells: a daughter cell with the same potential of the mother cell (stem) and the other with different potential able only to differentiate in a specialized line (progenitor). The progenitor cell are not able of dividing indefinitely (loss of staminality) and all of its progeny will differentiate into a single specialised cell or into diverse types of mature cells.

The present knowledge regarding stem cells can be divided into two groups: embryonic stem cells and adult stem cells [26].

The embryonic stem cells form the internal cellular mass (embryoblast) of the blastocysts and are capable of generating cells (both in-vitro and during the human embryogenesis) that maintain their capacity to differentiate in any tissue of the organism.

The adult stem cells, capable of being isolated during the fetal period in organs and during the neonatal period in the umbilical cord, are present in the adulthood in both the solid organ tissues and in the blood.

These cells maintain their ability to replicate and are able to replace differentiated cell loss so as to guarantee the physiological cell turn-over and the cell damage repair.

For a long time it had been hypothesized that the adult stem cells were capable of differentiating exclusively in mature cells of the tissues where they were hosted. That is, it was thought that the cells were organ specific or having a limited plasticity. However, the classic paradigm of the organ specific differentiation of the adult stem cells has recently been challenged, given the greater amount of evidence that has confirmed the persistence in these cells which possess a certain grade of plasticity or total potency [26, 27].

It was, also previously, believed that the endothelial cells and the smooth muscle cells could originate from different cellular precursors [28]. In fact, it was held that during embryogenesis the endothelial cells derived from two progenitors: the angioblast, capable of differentiating in mature endothelial cells and the haemoangioblast, capable of generating in both endothelial cells and common blood cells. Even in adults the existence of a medullar precursor has been documented. This stem cell has the characteristics of a haemoangioblast, which is responsible for the formation of blood and endothelial cells. This progenitor may

migrate from the stromal zone to the medullar vascular zone, and then procede to the differentiation steps towards the endothelial or haemopoietic precursor, which can occur initially in the medulla and subequently in the circulation[29]. The vascular muscle cells, distinct in vascular smooth muscle cells (muscle cells capable of presenting in more layers in the vascular walls of the arteries proximal to the heart) and in pericytes (muscle cells that present in mono-layers in the vascular walls of the more distal section of the arteriolar tree), can originate from diverse precursors: mesenchymal stem cells (in both embryonic and adult), embryonic cells of the neural crest, the epicardial embryonic cells and the endothelial progenitors [30]. Until a few years ago, the theory described above represented the most widely shared explanation for the origin of endothelial cells and smooth muscle cells of the arterial wall. The studies of Yamashita and coll. [31] , in contrast, have showed the existence of a new progenitor called "common vascular progenitor" capable of differentiating in both mature endothelial cells and in smooth muscle cells [31].

In particular, the exposure of the common vascular progenitor to Vascular Endothelial Growth Factor (VEGF) may promote its differentiation in endothelial cells, while the Platelet Derived Growth

Factor-BB (PDGF-BB) could stimulate differentiation in smooth muscle cells or in pericytes[31].

In this latter hypothesis, which dates back to the 1960's, the cells residing in the circulating blood of an individual adult are believed to contribute to the renewal of the vascular endothelium. It has been observed that the implantation of Dacron vascular prostheses in animals was followed by a rapid recovering of a cell layer which was identical to the vascular endothelium [32]. At that time, however, it was not yet clear if the endothelial recovering of the prosthesis had been the result of a migration of the mature endothelial cells adjacent to the prosthesis or had been derived from the progenitor cell differentiation, medullar or circulating, in the mature endothelial cells. Only later did it emerge that in the circulation it was possible to isolate cellular elements having noteworthy plasticity, with the capacity to actively proliferate and form endothelial capillary structures under favourable conditions [33,34]. In fact, $CD34^+$ stem cells of medullar derivation, which were transplanted in experimental animals previously fitted with Dacron vascular prostheses, were involved in the development of endothelial recovering for the lumen surface of the prostheses[35]. Through the markings of the transplanted cells, it was possible to

confirm that the endothelial recovering of the prostheses was obtained by the radication in loco of the transplanted medullar cells[35].

In addition, again in experimental animal models, it was demonstrated that the marrow cells can contribute not only to the recovering of the vascular material of the prosthesis, but also to the repairing of endothelial damage[36].

The circulating mobilisation of $CD133^{+}/VEGFR\text{-}2^{+}$ endothelial progenitors has been documented in humans exposed to significant vascular trauma, such as extensive burns or invasive coronary procedures[36]. In fact in these subjects the number of circulating endothelial progenitors immediately increased by more than 50-fold at a distance of 6-12 hr from the vascular trauma and return to baseline levels within 72 hr[36].

Important stimuli for the mobilisation of endothelial progenitors include not only particularly serious vascular traumas but also localized and extended ischemic tissue events[36,37,38,39]. For example, a significant increase in the number of endothelial progenitors in the acute phase of myocardial infarct has been observed[40,41,42,43,44,45].

Other studies carried out in different clinical situations have confirmed the capacity of endothelial cell progenitors, expanding numerically ex vivo, to regenerate ischemic tissue; including myocardium in subjects with acute myocardium infarct[46,47,48,49,50,51,52].

7. ENDOTHELIAL MICROPARTICLES: CAUSE AND EFFECT OF ENDOTHELIAL DAMAGE

Recently, new markers linked to vascular dysfunction have been identified: the endothelial microparticles (EMPs) [53,53,54,55,,56,57]. These markers have principally originated as a consequence of an activation process of the cellular membranes or by damage leading to apoptosis (fig2) [58,59,60,61,62,63].

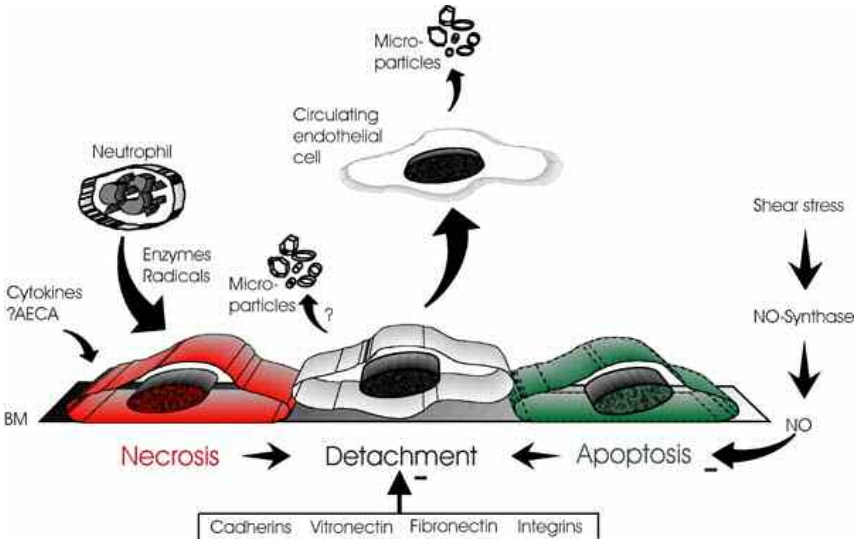


Fig 2. Endothelial microparticle formation scheme

Endothelial microparticle formation has been observed via numerous studies in both in-vivo and in-vitro[64,65,66,67,68].

In one of these studies, Jimenez e coll.[69] performed experiments in vitro inducing the activation of the endothelial cells by way of treatment with TNF-α e IL-1 and causing the release of EMPs.

Analogous results have also been obtained by Combes et al[70] where the release of EMPs was induced by treating the endothelial cells with the serum of patients with antiphospholipid syndrome. Furthermore, from a study by Laurence and coll. [71] the correlation between EMPs and apoptosis showed changes in the apoptic endothelial cells following exposure to TTP plasma.

According to the accepted definition present in literature , the EMPs are intact vesicles deriving from endothelial cell membranes, with their dimensions varying from 0,2 a 2,0 μm [72] and are characterized by antigen expression of specific surfaces of the endothelial cells, which document their endothelial origin. The profile of their phenotype can vary considerably depending on the type of process which the cells of origin have undergone: activation (predominant expression of antigen CD62E) or apoptosis (predominant expression of antigen CD31) [69].

Even though the role of EMP has not been clarified yet, strong evidence suggests that the EMPs are able to function as diffusible mediators of cytokines and adhesion molecules promoting the transduction of cellular activity [73]. In the study by Taraboletti and al.[74] revealed that the stimulation of HUVEC with angiogenesis growth factors (VEGF e FGF-2) led to the formation of EMPs rich

in metalloproteinases matrix and capillary structures, suggesting their potential role in angiogenesis. Furthermore, it has been demonstrated that the EMPs have procoagulant activity, defined through the action of platelet factor 3 and TF[75]. In the pro-thrombotic state, in fact, a study by Shet e coll. [76] has evidenced high values of TF-positive EMPs, which were strongly correlated with procoagulant activity. It is also noteworthy to report also the work by Jimenez and coll.[77] where a severe endothelial dysfunction in the aorta of mice after exposure to EMPs was evidenced. In this study it was demonstrated that the EMPs are capable of influencing the transduction pathway of NO, but not the expression of NO synthesis, with a consequential induction of endothelial dysfunction.

Further implications of the EMPs in the pathophysiology reside in their pro-inflammatory activity and their tendency to bind the activated monocytes. In the work by Boulanger and coll.[78]in fact , it has been demonstrated that the EMPs induce the release of cytokines (TNF-α e IL-1β) with the consequential pacrine and autocrine activations of the monocytes and the endothelium.

8. THE CREATION OF A PULMONARY HYPERTENSION DATABASE AND THE CORRELATION BETWEEN DISEASE GRADING AND INFLAMMATORY INDEXES

8.1 Materials and methods

Our research was, above all, aimed at identifying the patients affected by pulmonary hypertension (PH), who had been admitted to the Padua University Hospital, and re-evaluating their clinical diagnoses on the basis of new classifications adopted at The 2003 World Symposium on Pulmonary Arterial Hypertension held in Venice, Italy.

We create a database of patients, updated according to the new nomenclature and on the basis of collected data we carry out our study.

With this goal in mind, we obtained a lists of patients from the administrative offices, admitted between 2000 and 2006 at University Padua Hospital and discharged with the code 4160 (primary pulmonary hypertension) or with the code 4168 (other forms of pulmonary hypertension) for the disease classification ICD-9-CM[79].

A *Microsoft Excel file* of 255 patients was created which included: the first and last name of the patient, date of birth, type

of admission, medical chart number, dates of admission and discharge, the departments of admission and discharge, the principal diagnosis and 6 secondary diagnoses, the names of up the departments in which the patient was moved during the hospital stay.

According the ICD-9-CM codes, which specifically referred to the primary form, it was impossible to distinguish between the familial form and the idiopathic form, since the two were included in the same code. The recoding was made on the basis of a thorough evaluation of all the information available in the clinical charts.

The other important evaluation that was done at the time of the medical chart analysis concerned the secondary form that the international classification identifies generically as other forms of pulmonary hypertension (PH) with the code 4168. The new classification of PH distinguishes these latter into 5 classes.

To fulfil the privacy low, the names of the patients were transferred from the excel file to an access file where the data of the patients were registered. Furthermore, we created excel files for the numerical values to perform statistical analysis.

For each medical chart , we identified these most significant data: main diagnoses and up to 3 comorbities; relevant family

history;remote pathological anamnesis; NYHA classes at diagnosis and after treatment; haematochemical exams including blood cell count at admission and after therapy; ESR; serum protein electrophoresis; plasma level of fibrinogen; C-reactive protein, uric acid, albumin; electrocardiographic data signalling the absence or presence of right ventricular hypertrophy; echocardiographic data with a particular attention to the severity of pulmonary hypertension, dimension and dynamics of right cardiac cavities; ABG (arterial blood gas) values at baseline and after therapy; pulmonary functioning test; 6 minute walking test; right cardiac catherization data and finally medical treatment performed.

Two hundreds fifty-five patients were selected(fig 3). Thirty-five (14%) were excluded because the clinical diagnosis of pulmonary hypertension didn't satisfy the definition of Venice Classification. Thirthy-six patients were affected by PAH.

In our database the total number of patients affected by other forms of PH was 184 subjects.

Since we had limited our research to forms of PAH, patients with other forms were excluded.

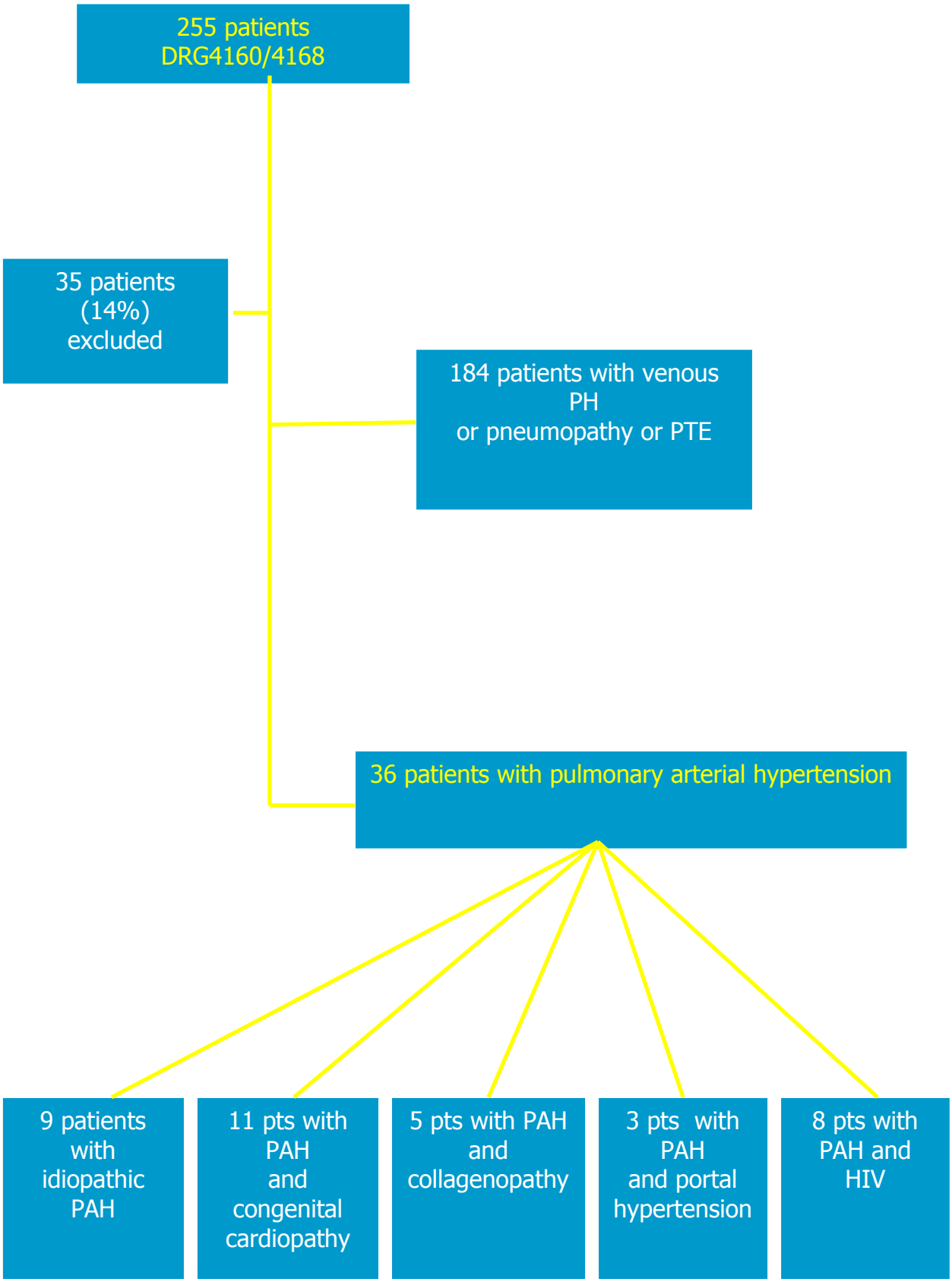


Fig 3. Diagram of patient database selected by ICD-9-CM code.

Idiopathic arterial hypertension

During the study period (2000-2006) 2±1 cases of idiopathic PAH for year were reported.

Two of the nine patients with idiopathic PH presented positive family hystory for PH and they were sent for genetic testing at the University of Bologna and considered affected by familial PH.

The ratio men/women was 1.6:1, confirming the prevalence of these disease in the female sex.

The average age of the subjects of both sexes at diagnosis was 54 ±1 year. Only a boy of 12 years, showed an idiopathic PAH form, at echo and cardiac catheterization.

Body mass index, calculated from reported chart data on height and weight resulted normal with values of 25±4. (tab 2)

The time interval between onset of symptoms and the final diagnosis was 12±6 months in 5 subjects, and 24±6 months in the remaining 4 patients.

The clinical presentation was highly variable. Dyspnoea during exercise and at rest was the symptom most frequently reported. Moreover 3 patients suffered episodes of syncope. Angina at rest and after exercise was reported by 4 patients. Aspecific symptoms

which included asthenia and abdominal discomfort were also reported by all of the subjects.

At clinical examination, most of the subjects presented severe symptoms at diagnosis, with none of the patients in NYHA I class, 6 patients in NYHA classes II/III and 3 patients in NYHA class IV (tab2).

The mean values of the pulmonary artery systolic pressure values were obtained by Doppler method from tricuspid regurgitation[80,81,82] was 53±15 mmHg. The arterial pulmonary systolic pressure values were higher in subjects in the advanced NYAH classes.

Five patients showed at ECG right ventricular hypertrophy.

Normal value of ABG analysis were present at rest and at room temperature in all cases.

The distance covered during the 6MW test showed a correlation with the NYHA classes of the patients: 6 subjects in NYHA classes II and III travelled an average distance of 360 meters, 3 patients in NYHA class IV exhibited a capacity to cover a much shorter distance of 190 meters.

The respiratory function tests were in the normal range.

At cardiac catherization the mean values was PAP values was 56 ±15 mmHg and normal capillary wedge pressures. and pulmonary

vascular resistance of 18±5 UI. Pharmacological tests [82] with NO inhalators or intravenously delivered prostacyclin were carried out in 4 out of 5 subjects examined and 2 subjects were responders.

All the patients were treated with oral anticoagulants. Calcium antagonists (nifedipin, ditiazem or amlodipine) were taken by 3 patients. Seven patients were in therapy with receptor antagonists of endothelin (bosentan). In three patients epoprostenol ev was administered.

Six of the nine patients had repeated admissions to hospital. Considering the lack of homogeneity in the tests performed during readmission, it was not possible to carry out analyses on: response to therapy, disease evolution and patient survival.

Table 2. Parameters of patients with idiopathic pulmonary hypertension

Age	54 years ± 12 months
Sex	Men/Women 1.6:1
NYHA classes at diagnosis	6 pt in NYHA classes II-III 3 pt. in NYHA classes II-IV
Time between symptom onset and diagnosis	5 pt 12±6 months 4 pt 24±6 months.
ECG	5 pt right ventricular hypertrophy
Eco PAPs	53±15 mmHg.
6MW test	Median distance covered 306 meters
Median PAP	56±15 mmHg
PVR (pulmonary vascular resistance)	18±5 UI

Associated forms of pulmonary arterial hypertension

Congenital heart disease

Our associated PAH cases consists of 27 patients of which eleven presented congenital heart disease, five collagen disease, eight AIDS, three portal hypertension correlated to hepatic pathologies (two affected with hepatic cirrhosis and one had Budd-Chiari syndrome). No cases of secondary farmacologic induced pulmonary arterial hypertension could be identified.

Eleven patients affected by congenital heart disease had median age of 25 years±6 months. The ratio men/ women was 1.5:1.

Six patients had an interventricular septum defect: three isolated, one associated with patent ductus areriosus and atrial septum fossa ovale type defect; one patient had a defect of atrial-ventricular septum; a single case of pulmonary atresia with ventricular septal defect and systemic pulmonary plurifocal vascolarization, two subjects had a common truncus arteriosus, one had an non obstructive total venous anomalus pulmonary vein drainage into the coronary sinus.

The mean values of the PAPs at echocardiogram was 55±12 mmHg. At cardiac catherization the mean value of PAP was 56 ±15

mmHg, normal capillary wedge pressure along with pulmonary vascular resistance 16±8 UI.

All patients of this series were under therapy with antagonist receptors of endothelin *(bosentan*).

Other forms

Five patients with PAH associated with a collagen disease had a mean age of 55 years±14 months. The ratio men/women was 1:2.

In this group of patients the most frequent disease was scleroderma (two of which had the diffused form and one had the limited form (CREST)). One patient was affected by lupus; one presented an undeterminated mixed connective tissue disease.

All patients were in NYHA classes II or III. Most of the subjects showed the phenomenon of Raynaud. The mean PAP values were 65±5 mmHg. Invasive haemodynamic investigation was carried out in 3 of the patients from the group.

In our analyses we considered patients with all the forms of pulmonary hypertension, included in the first group of the International Classification held in Venice 2003, eventhough we recognized that patients with asociated connective tissue disorders could present higher indexes of inflammation.

Data are presented as means and standard error of the mean (SEM). In order to compare the above listed variables between the PH patients and the healthy controls, the Student's t-test was performed on the parametric variables. While the Wilcoxon test was performed on the non-parametric variables. The Pearson and Spearman correlation analyses were utilized to quantify the statistical correlations, respectively among the parametric and non-parametric variables. ANOVA was employed to compare the levels of the various phlogistic indexes among the patients grouped in the fourth NYHA class.

8.2 Results

The analysis of the data of the 36 patients with idiopathic or associated PAH and the data obtained from the 36 healthy controls, showed:

1. The patients affected by PAH compared to healthy control subjects had white blood cell counts of 6700±113/mm^3 vs 6360±156/mm^3 and ESR levels of 21±2.5 mm vs 16±2.7 mm respectively. However, a statistically significant difference was found ($p<0.05$) for CRP (4.7±0.09 mg/l vs 1,6±0.04 mg/l), fibrinogen (465±53 mg/dl vs 319±66 mg/dl), uric acid (5,1±0.8 mg/dl vs 3,2±0.7 mg/dl), which were higher in patients with pulmonary arterial hypertension (fig 4). On the other hand, the levels of albumin was lower in patients with pulmonary arterial hypertension than in healthy controls (3.1±0.8 g/dl vs 4.1±1.1 g/dl, $p=0.026$).

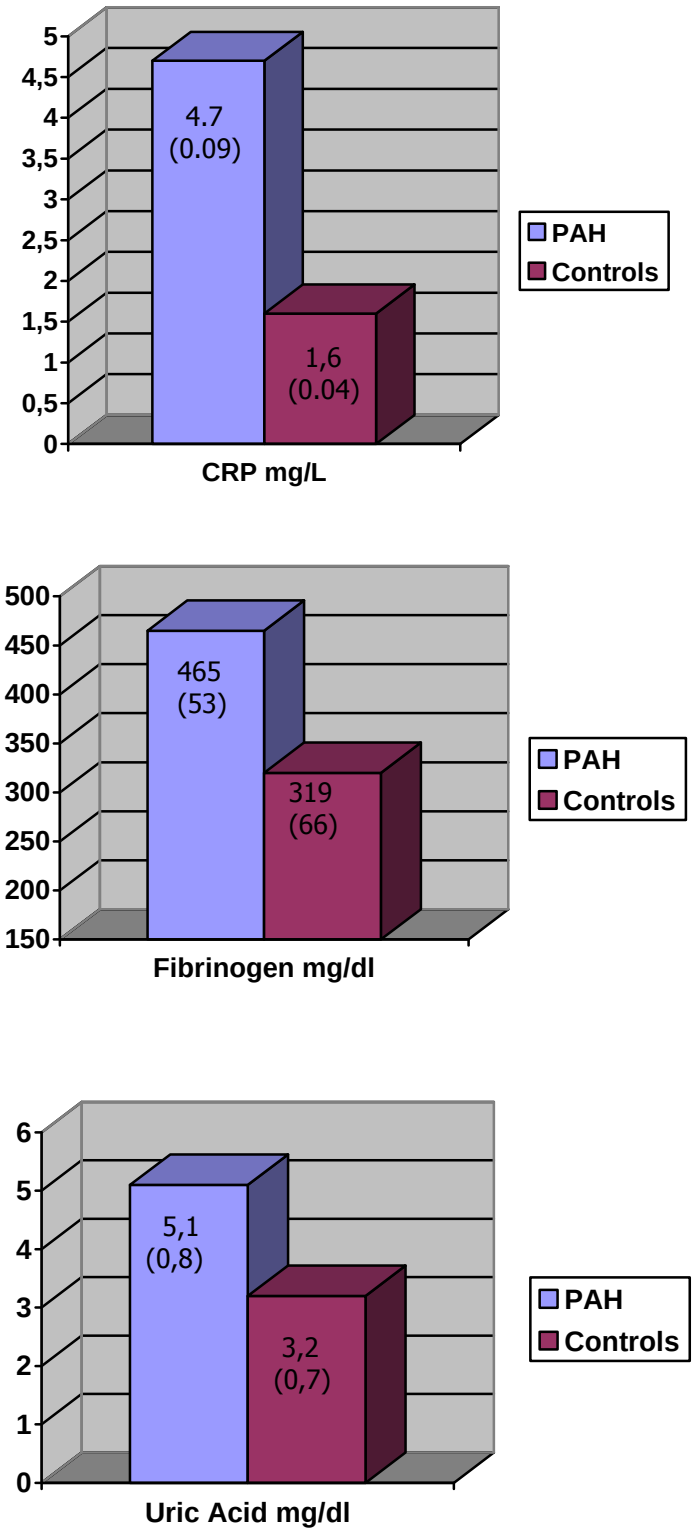


Figure 4. CRP, fibrinogen and uric acid blood levels in patients with pulmonary arterial hypertension (PAH) and controls. Data are presented as means plus standard error of the mean (SEM).

2. Patients in the NYHA III and IV classes vs those subjects in the NYHA classes I and II, showed the highest levels of inflammatory parameters: CRP (6.9±0.08 mg/l vs 2.8±0.05 mg/l), fibrinogen (513±69 mg/dl vs 408±88 mg/dl) and uric acid (7.6±1.1 mg/dl vs 2.6±1.4 mg/dl). Non significant differences were observed in the other inflammatory indexes.

3. The previous results were confirmed by the 6MWT; subjects with less distance covered during 6MWT had higher levels of: CRP ($r = -0.47$, $p=0.002$), fibrinogen ($r=-0.33$, $p=0.009$) and uric acid ($r=-0.36$, $p=0.008$). On the contrary ESR, white blood cell count levels and albumin levels were not significantly associated with the 6MW test results.

8.3 Discussion

A series of relevant observations could be drawn from the analysis of clinical notes of patients with PH selected in our database.

Non specific symptoms characterized patients with pulmonary hypertension. In fact the diagnosis needs a quantitative evaluation by echocardiogram or cardiac catheterization. Moreover different disease can produce the same haemodynamic findings. All these facts produce bias in clinical diagnosis reported in the charts and may justify the relative low number of patients recorded.

The nosologic modifications introduced at the Venice Convention produced a significant improvement, because endorsed the concept that clinical conditions with different ethiology but with the same pathological structural alterations could be grouped togheter.

According to the Venice Classification we structured our database enrolling patients with different ethiology but with the same disease.

From the result of our analysis we can state that patients with PAH present a sub-clinical activation of the inflammatory cascade, which could be related to the grade of pulmonary vascular disease.

The role of uric acid in inflammation process is controversial. However we can confirm the increase of uric acid blood levels in

patients with pulmonary arterial hypertension, previously reported in others studies. Probably elevated blood levels of uric acid is the consequence of an impaired oxidative metabolism of the tissue, with increased degradation of adenine nucleotides such as ATP, as a consequence of both systemic and pulmonary inflammation.

Even if the increase in CRP and fibrinogen levels can not represent a pathognomonic markers for the diagnosis of PAH, the use of these indexes could improve our ability to stratify the severity of pulmonary vascular damage in patients with PAH.

The parameters considered are easily performed in everyday clinical setting and significantly correlate with the severity of the disease. The results of our study eventhough carried out in a small number of patients suggest that they may be of help to clinical decision-making for specialist dealing with PAH patients.

9. THE CORRELATION BETWEEN PULMONARY ARTERIAL HYPERTENSION AND NEW INDEXES OF ENDOTHELIUM DAMAGE AND REPAIR.

9.1 Population

The study here, was carried out on a group of 15 patients affected by PAH from the Martini Hospital of Turin, the Sant'Andrea Hospital of Vercelli and the San Luigi Gonzaga Hospital of Orbassano, Italy (table3). Our case study was heterogeneous for the different forms of PAH. All of the patients signed informed consensus forms before participating in the study.

The subjects in examination were all under therapy with endothelin receptor antagonists.

All patients had a negative history for cardiovascular disease, dyslipidemia, diabetes, smoking and systemic hypertension. A carotid echo-Doppler scan was performed on all patients and showed normal values of intimal-medial thickness. A brachial arterial flow-mediated vasodilation after reactive hyperemia was assessed by ultrasonography and resulted normal in all patients.

In the table (tab3) below the patients' principal characteristics examined are reported: type of PAH, NYHA class, 6MW test results and PAP values at the start of the study.

Table 3. NYHA class and values of PAPs and 6MWT in patients with pulmonary arterial hypertension.

PATIENTS	PAH	NYHA CLASSES	6MW test (*meters)*	PAPs at echo-Doppler (*mmHg)*
A. T.	IPAH	II	270	76
Q. L.	IPAH	III	300	90
D.P. R..	IAPI	II	310	82
M. M.	IAPI	III	400	115
G. V.	SSC e IAP	III	360	69
M. A.	SSC e IAP	II	420	105
Z. G.	SSC e IAP	II	350	96
C. S.	SSC e IAP	III	300	88
P. M. P.	LES e IAP	II	290	84
C. S.	CBP e IAP	II	350	75
O. M.	DIV e IAP	I	305	97
D. F. C.	DIV e IAP	II	265	89
P. F.	DIV+DIA e IAP	II	300	102
P. E.	DIV+DIA e IAP	II	450	69
D. S. L.	DIV+DA e IAP	II	500	87

.

Fifteen patients affected by PAH were compared with 15 healthy subjects matched for sex and age.

9.2 Materials and Methods

Fasting blood samples were taken between 8:00 a.m. and 10:00 a.m. The measurements of the endothelial microparticles were performed as described by Jimenez e coll.[69]. In short, from each patient a venous blood sample was taken and the obtained blood underwent sequential centrifugation so to obtain plasma poor in platelets (PPP). Every sample of PPP was then incubated for 30 minutes at 4°C with fluorescent antibodies: anti-CD31 united with phycoerythrin fluorochrome (PE) and anti-CD42a united with fluorochrome *fluorescein isothiocyanate* (FITC). At this point , a cytofluorimetric analysis was carried out to identify from the sub-population of the MPs: the presence of antigen 31 on the surface of the membrane , absence of antigen 42 and dimension less than 1,5 μm. This operation was conducted following a dimensional gate via the use of calibration spheres for cytofluorimeter at a known diameter (fig5).

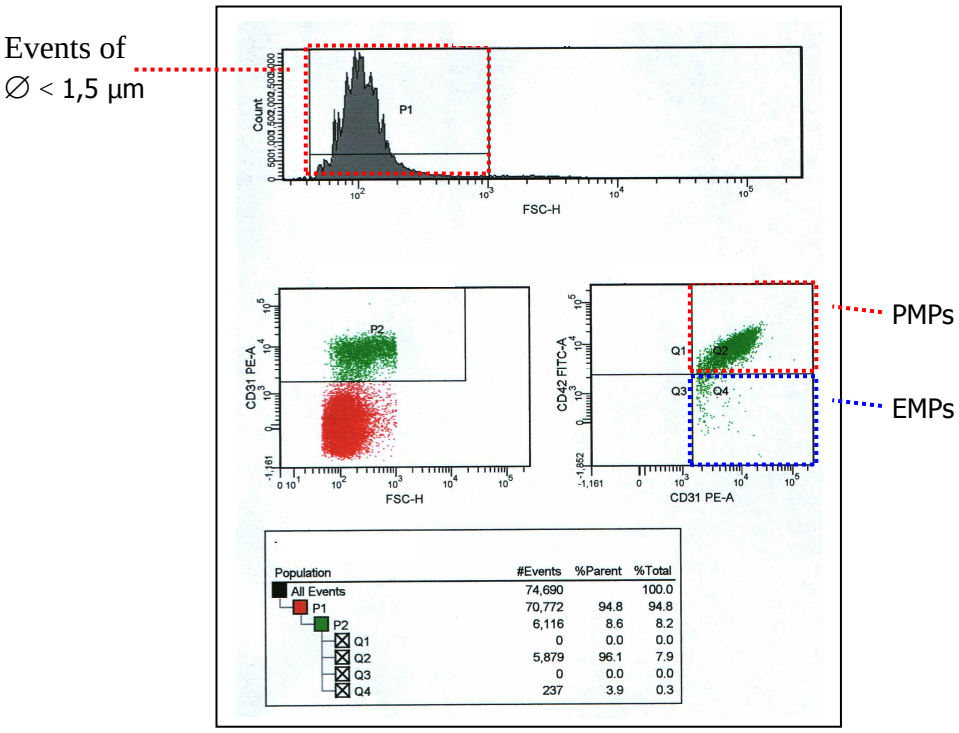


Fig 5. Cytofluorimetric analysis of the EMPs

To determine the number of endothelial progenitors a blood sample was taken, from every participant in the study. EDTA was used as an anticoagulant in the test tube blood samples. Each sample of whole blood underwent a centrifugation on density gradient so as to separate the mononuclear cells (lymphocytes e monocytes). After sequential washing with PBS, so to eliminate the eventual platelet contamination, the obtained cells were incubated at 4°C for 30 minutes with antibodies anti-CD34-FITC and anti-KDR-PE. After

which, each sample underwent a cytofluormetric analysis so to individualize endothelial progenitors characterized by the expression of CD34 and KDR (fig 6).

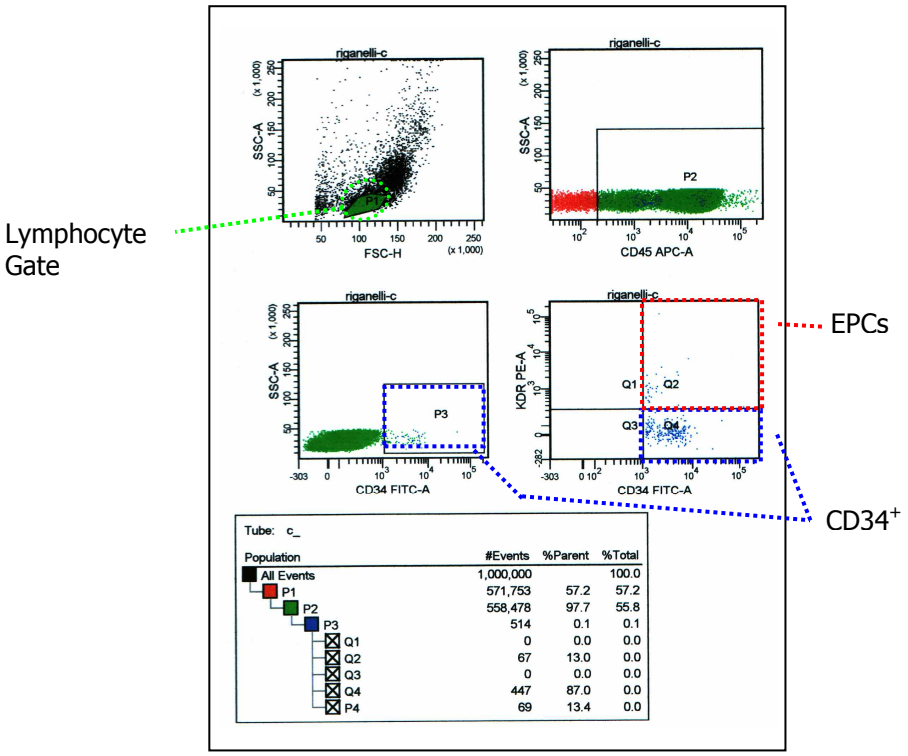


Fig 6. Cytofluorimetric analysis of the endothelial progenitors.

The statistical analysis was performed with SPSS 15.0. The statistical significance was considered for the values of $p<0.05$. For the correlation among the continuous variables this was done utilizing Pearson's correlation coefficient. Regarding the comparison among the categorical variables the X^2 was used. The comparison among continuous variables was performed with the Student's t-test for the parametric variables and the Wilcoxon for the non-

parametric variables. The values have been expressed as median ± SEM.

9.3 Results

The results from this study show that patients with PAH have a higher number/µl of endothelial microparticles (835.8±15.7 *vs* 710.9±31.7; p<0.01) and a lower number/ml of endothelial progenitors (423.9±7.4 *vs* 498.2±26.2; p<0.01), compared to healthy controls (fig 7-8).

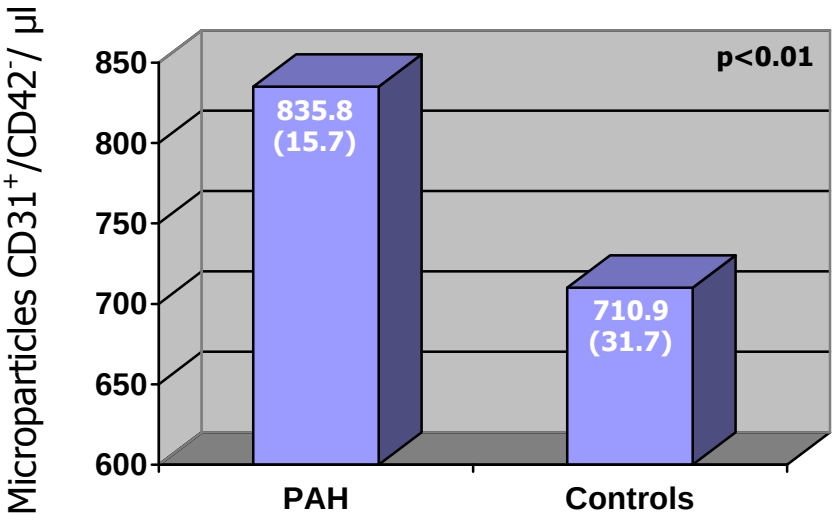


Figure 7.Number of Microparticles $CD31^{+}/CD42^{-}$ /µl in subjects with PAH: pulmunary arterial hypertension and healthy controls. Data are presented as mean and SEM.

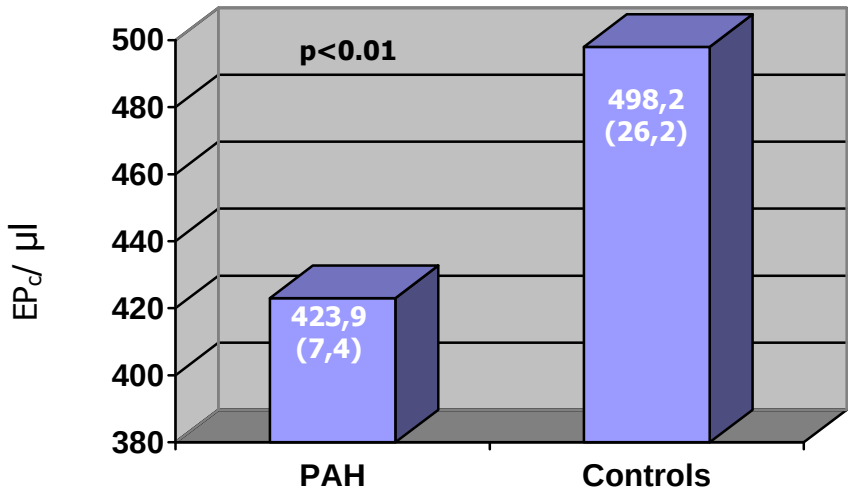


Figure 8.Number of EPc/µl in subjects with PAH: pulmonary arterial Hypertension and healthy controls. Data are presented as mean and SEM.

The correlation analysis performed in the entire cohort between the number of endothelial microparticles and the PAP levels assessed at echocardiography demonstrates that there exists a significantly positive relationship between these two variables (r=0.609; p=0.004). Figure 9 shows the increase of endothelial microparticles for increasing tertiles of PAPs (1st tertile: < 35 mmHg, 2nd tertile 35-85 mmHg, 3d tertile >85 mmHg).

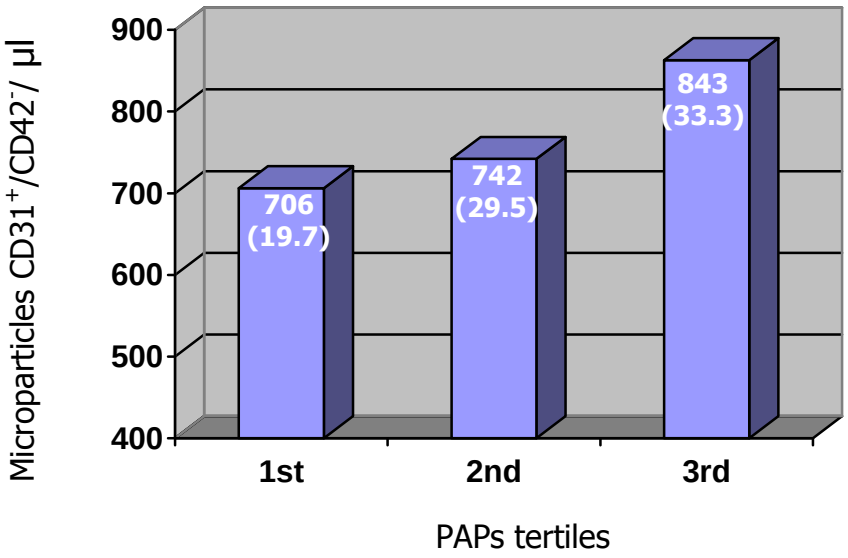


Figure 9 Increase of endothelial microparticles for increasing tertiles of PAPs (1st tertile: < 35 mmHg, 2nd tertile 35-85 mmHg, 3d tertile >85 mmHg). Data are presented as mean and SEM.

Furthermore, the correlation between the number of circulating progenitors of the endothelial cells and the PAP values has shown a negative correlatioon (r=-0.463; p=0.05). Figure 10 shows the decrease of endothelial progenitor cells for increasing tertiles of PAPs (1st tertile: < 35 mmHg, 2nd tertile 35-85 mmHg, 3rd tertile >85 mmHg).

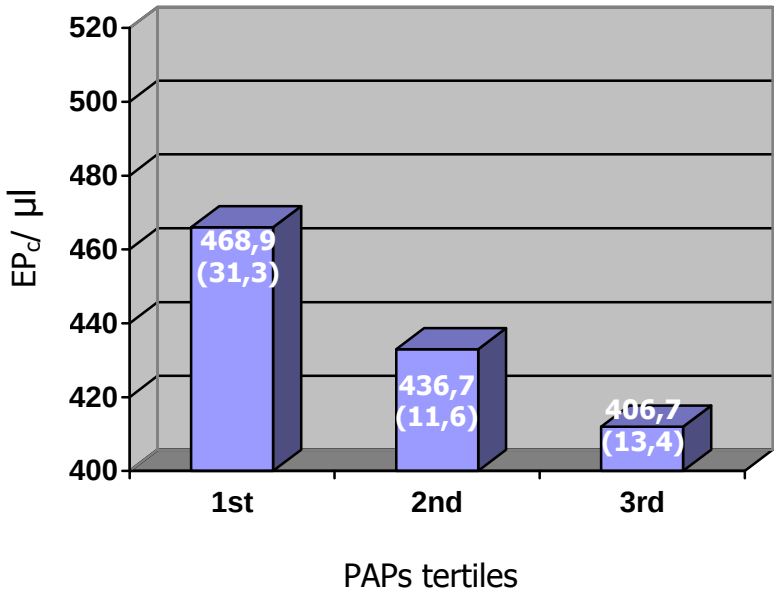


Figure 9. Decrease of endothelial progenitors cells for increasing tertiles of PAPs (1st tertile: < 35 mmHg, 2nd tertile 35-85 mmHg, 3d tertile >85 mmHg). Data are presented as mean and SEM.

A negative correlation between the number of microparticles and the 6MW test results was found (r=-0.573, p=0.001). Figure 11 shows the increase of endothelial microparticles for decreasing tertiles of distance travelled at the 6MWT (1st tertile >600 meters, 2nd tertile 350-600 meters, 3rd tertile <350 meters).

On the other hand, the number of endothelial progeniors cells decreases with a reduction in the results from the 6MW test (r=0.444; p=0.014). Figure 12 shows the decrease of endothelial progenitor cells for decreasing tertiles of distance travelled at the

6MWT (1st tertile >600 meters, 2nd 350-600 meters, 3rd <350 meters).

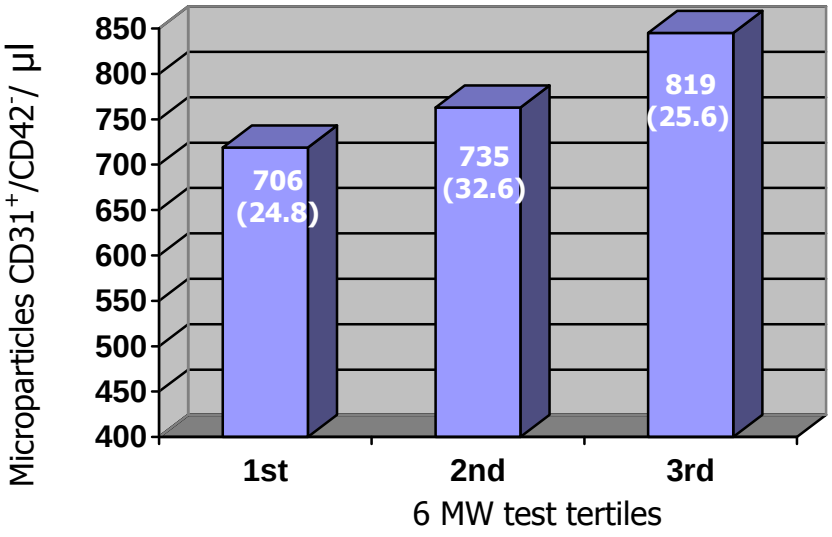


Figure 11. Increase of endothelial microparticles for decreasing tertiles of distance travelled at the 6MWT (1st tertile >600 meters, 2nd tertile 350-600 meters, 3rd tertile <350 meters).

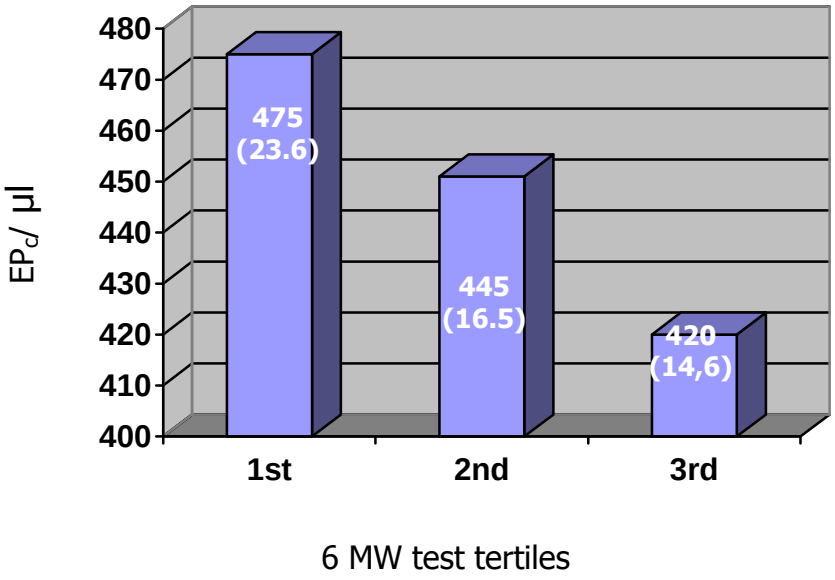


Figure 12. Decrease of endothelial progenitor cells for decreasing tertiles of distance travelled at the 6MWT (1st tertile >600 meters, 2nd tertile 350-600 meters, 3rd tertile<350 meters).

9.4 Discussion

In our study we report an increase in the number of circulating endothelial microparticles and endothelial progenitor cells in patients with pulmonary arterial hypertension when compared to normal volunteers. We have shown these markers correlate with the pulmonary artery pressure and the exercise capacity of the individual.

We hypothesized that the shear stress could result in the shedding of the involved endothelial cells, resulting in an increase of circulating endothelial microparticles.

Although we were unable to identify the precise site of origin of the endothelial microparticles in our patients, we suggest that the only likely site of origin is the pulmonary vasculature. This hypotesis is based on: 1) the microparticles demonstrate markers $CD31^{+}/CD42^{-}$ of endothelial origin (the pulmonary vasculature has the greatest microvascular surface area in the body), 2) the number of microparticles correlate significantly with the severity of pulmonary hypertension, 3) in patients with PAH the pulmonary vascular bed is the predominant site of pathology, 4) in our patients the inclusion criteria exluded the presence of pathologies producing systemic endothelial vascular damage .

On the other hand, we were unable to determine the homing and differentiation of the endothelial progenitors cells, but we can affirm that their number is impaired in patients with pulmonary vascular disease.

Because we did not have lung biopsy samples in our patients, it was not possible to correlate the angiogenetic properties of endothelial progenitor cell with the presence of plexiform lesions.

However it was clear that these cells could play an important role in the pathobiology of pulmonary arterial hypertension.

From the results of our study we think that endothelial microparticles and endothelial progenitors can serve as a surrogate marker of the disease process, and can provide a test for early diagnosis of severe pulmonary hypertension.

10 CONCLUSIONS

Pulmonary arterial hypertension is listed among the rare diseases, and is characterized by vascular obstruction, leading to increased pulmonary vascular resistence and right side heart failure.

It occurs in women more often than man and, most important, has a mean age at diagnosis of 36 years and is usually fatal within 3 years without therapy. Modern treatment has markedly improved physical function and has extended survival.

We still do not understand what initiate the disease and what allows it to progress. Recently genetic factors has been indentify as key player in the development of this multifactorial disease.

Our interest in the inflammatory biomarkers reflect the current momentum and excitement surrounding research on PAH.

The aim of our research was to determine the role of inflammation in the pathobiology of the disease through the assessment of inflammatory biomarkers and to indentify them as a possible clinical useful biomarkers that allow non invasive diagnosis and monitoring of PAH.

The balance between endothelial damage and repair is essential for the maintenance of vascular homeostasis. We think that the endothelial microparticles $CD31^{+}/CD42^{-}$ represent a new and valid

indicator for damage produced at the level of the pulmonary vasculature. On the other hand, greater evidence seems to point to the fact that endothelial damage is a condition essential, but not sufficient for the deterioration of the vascular function. It is clear that the latter is the result not only of the vascular damage but also to the repair mechanisms.

Currently, many evidence support that hypotesis that endothelial progenitor cells play a key role in the repair function of damaged endothelium. Taken this as it may, all the conditions that are capable of influencing the number or the function of endothelial progenitor cells are significantly involved in the onset of vascular damage.

The results of this study demonstrate that PAH is the result of the imbalance between damage and repair mechanisms, which together guarantee normal vascular function: increased numbers of endothelial microparticles as results of vascular damage and decreased number endothelial progenitors cells as alteration of repair mechanism.

In other words, PAH produces a fragmentation of mature endothelial cells, increases formation of endothelial microparticles and reduces the number of circulating endothelial progenitors. This

constitutes the functional expression of a vascular dysfunction which in turn determines a structural and biological reshaping of the pulmonary vascular tree and furthermore, *also involves complex systemic homeostatic mechanisms.*

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